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SECTION MEETINGS

ILLINOIS	
University of Chicago	October 27, 1942
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SOUTHERN CALIFORNIA	
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WESTERN NEW YORK	
University of Rochester	October 17, 1942

13894 P

Influence of Repeated Daily Exposure to Low Barometric Pressure on Urine Output.*

HERBERT SILVETTE. (Introduced by H. E. Jordan.)
From the Pharmacological Laboratory, University of Virginia Medical School.

Armstrong[1] has reported that humans subjected daily to a simulated altitude of 12,000 feet in a low-pressure chamber excreted on the average 100% more urine than at sea level. An even more marked polyuria has been invariably observed in white rats maintained at a level of 15,000 feet for periods of 3 hr;[2] though in the case of anesthetized dogs breathing low-oxygen gas mixtures, Van Liere *et al.*[3] and Toth[4] have observed oliguria to occur more frequently than polyuria. The present experiments were undertaken to determine whether, on repeated daily exposure to low barometric pressure, this polyuria would be maintained over a long period of time, or whether acclimitization and consequent normal urine output would result.

Healthy male white rats, weighing from 150 to 200 g, were maintained on a stock diet of Purina Dog Chow and liberal amounts of greens, with water allowed *ad libitum*. Food, but not water, was removed from the 12 animals comprising an experimental group 12 hr before the start of an experiment, and the following morning the rats were placed in wire-mesh metabolism cages set over graduated cylinders in a specially-designed low-pressure chamber (capacity 500 liters). The chamber was evacuated to a pressure of

* This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council of Pharmacy and Chemistry, American Medical Association.

1 Armstrong, H. G., *Principles and Practice of Aviation Medicine*, Baltimore, 1939, p. 284.

2 Silvette, H., unpublished observations.

3 Van Liere, E. J., Parker, H. S., Crisler, G. R., and Hall, J. E., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 479.

4 Toth, L. A., *Am. J. Physiol.*, 1937, **119**, 127.

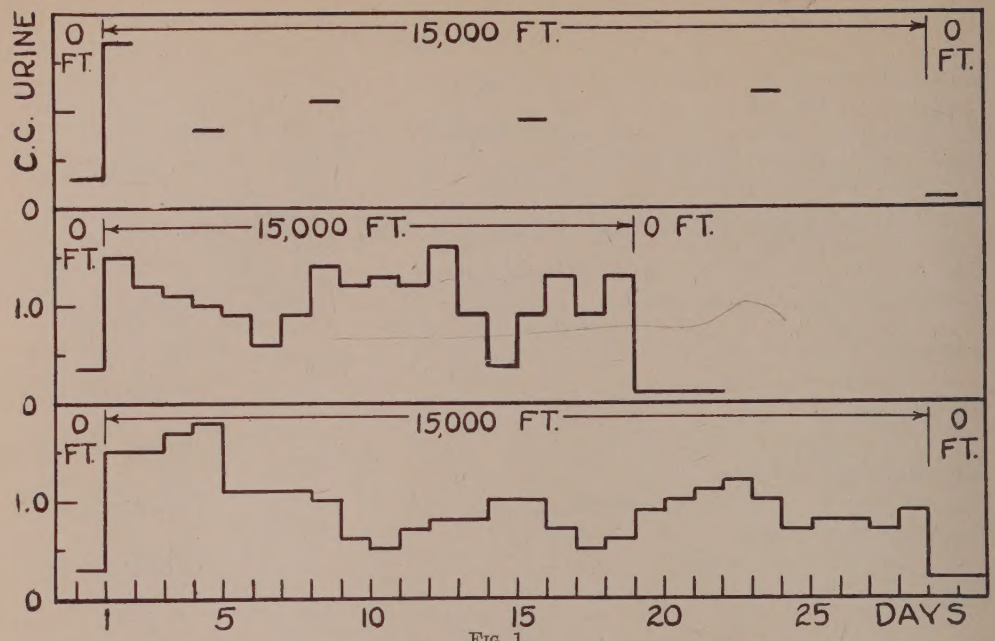

FIG. 1.

Effect of low barometric pressure on urine output of white rats exposed for 3 hours daily to an equivalent altitude of 15,000 feet (428.8 mm Hg).

Bottom curve, group I (12 animals); middle curve, group II (12 animals); top curve, group III (12 animals, *exposed daily* to 15,000 foot altitude, but daily urine collection not made); see text.

428.8 mm Hg (15,000 feet altitude equivalent) and maintained at this level for 3 hr, at the end of which time air was admitted; the animals removed; and the urine volume read and recorded. The temperature of the chamber was not precisely controlled; in each experiment, however, a thermometer inserted through the wall was observed to rise, in the course of the 3-hr period, from an initial 23° to a final 28°C.

A group of 36 animals, placed in metabolism cages for 3 hr at room pressure, excreted an average of 0.4 cc urine per 100 g body weight. The same animals, maintained for 3 hr at 15,000 feet, excreted on the average 1.7 cc, an increase of over 300%.

Three other groups of animals (12 in a group) were run in the low pressure chamber at 15,000 feet altitude equivalent for 3 hr a day for 29, 19 and 29 days respectively. The daily urinary output of the first group, expressed in cc urine per 100 g body weight per 3-hr metabolism period, is graphically shown in Fig. 1. It will be seen that the great initial diuretic response to high altitudes was not maintained. A degree of acclimitization seemed to occur; but at no time did the urine output for the 15,000 feet metabolic period fall to that observed at room pressure, and the average urinary output of the twelve rats throughout the 31 days of the experiment was 1.0 cc compared to 0.35 cc for the same animals at room pressure.

The cyclic character of the curve may perhaps be due to meteorological conditions prevailing during the course of the experiment, for a second group of animals, tested separately over the same period of time, showed a similar cyclic response of alternate profuse and less marked polyuria. In this second group, run for 17 days, no acclimitization to the high altitude was observed, so far as urine output was concerned. When both these groups were tested at room pressure at the end of the experimental period, the urine output was found to approximate that of normal animals never placed in the low-pressure chamber.

A third group of animals, similarly treated, was sacrificed at the end of 29 days, and the kidneys, adrenals, and other organs and tissues removed for weighing and histological examination. The average weight of the kidneys in the group run daily at 15,000 feet was 945 ± 14 mg (probable error of the average) per 100 g body weight; while the average weight of the kidneys in a series of normal animals, under precisely similar dietary and other conditions, save that these control rats were kept continually at room pressure, was 870 ± 14 mg. The kidneys of the experimental animals were 75 mg heavier than those of the controls, and this difference is statistically significant (probable error of the difference, ± 20).

The polyuric response of white rats to daily 3-hr exposure to an altitude of 15,000 feet is thus seen to be accompanied by renal hypertrophy. It should be emphasized that the conditions of the experiment were not critical. The animals emerged from each daily sojourn in the low-pressure chamber with no symptoms of strain or malaise, and during the course of the protracted experiment they gained weight normally and remained in excellent health. Nevertheless, these conditions (daily 3-hr exposure at 15,000 feet) were apparently severe enough to lead not only to hypertrophy of the kidneys, but also to hypertrophy of the adrenal glands and lymphoid tissue, findings which will be reported later by Dr. J. E. Kindred of the Department of Anatomy in another connection.

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The Search for a Physiologically Active Substance in Calf Thymus Tissue.

H. A. HOSTER, with the Technical Assistance of J. B. Welch. (Introduced by C. A. Doan.)

From the Division of Research, Department of Medicine, Ohio State University.

During 1940, Bomskov and associates published a series of articles[1] in which they described a new hormone obtained by extracting fresh calf thymus tissue with lipoid solvents. Complete details of the method of preparation were not given. The hormone mentioned is described as the mediator of the pituitary diabetogenic effect, the effector of blood sugar elevation, and the initiator of intense growth. Involution of male gonads and adnexa and inhibition of the effect of the thyrotropic hormone follow its use. Sensitivity to chloroform is increased. The hormone is said to be transported by an increased number of circulating lymphocytes and to be detectable in small quantities of urine. In the tadpole growth is stimulated but metamorphosis is retarded. Greatest secretion and consequent urinary excretion is said to occur in those clinical states which exhibit most marked lymphocytosis, in carcinoma, and in Hodgkin's Disease.

Very recently Wells and associates[2] reported their inability to confirm Bomskov's results. Using the rat and Carneau pigeon as experimental animals they failed to observe any significant blood sugar or liver glycogen variations, ketonemia, or any constant leucocytosis following injection of their extracts. Neither tissue equivalents of extracts used nor nonspecific control tissue extracts were mentioned by these investigators.

The present study was begun two years ago in an attempt to establish the presence of a physiologically active, lipoid solvent soluble

[1] *a.* Bomskov, C., and Sladovic, L., *Deut. Med. Wchnschr.*, 1940, **66**, 589; *b.* Bomskov, C., and Holscher, B., *Z. Klin. Med.*, 1940, **137**, 745; *c.* Bomskov, C., and Sladovic, L., *Pflüger's Arch.*, 1940, **243**, 611; *d.* Bomskov, C., and Brachat, F., *Endok.*, 1940, **23**, 145; *e.* Bomskov, C., and Karl-Heinz, K., *Pflüger's Arch.*, 1940, **243**, 623; *f.* Bomskov, C., *Pflüger's Arch.*, 1940, **244**, 246; *g.* Bomskov, C., *Endok.*, 1941, **23**, 239; *h.* Bomskov, C., *Endok.*, 1941, **23**, 225.

[2] Wells, B. B., Riddle, O., Marvin, H. N., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 473.

substance in thymus tissue. Several months after this work was initiated the first paper by Bomskov *et al.*[1] came to our notice and it was apparent that our investigations were to a great extent parallel in purpose and method.

Preparation of the Extract. Approximately 125 lbs. (57 kg) of fresh frozen calf thymus tissue was finely ground in 10-lb. lots and extracted using one liter of 95% ethyl alcohol or one liter of acetone or one liter of equal parts of alcohol and acetone per lb. of tissue. Seventy-five pounds of kidney and lung tissue were similarly extracted for use as control tissue extracts. As a further control we used appropriate quantities of the vehicle, corn oil, in which the extracted residues were dissolved. The initial extraction mixtures of tissue and alcohol were agitated for 6 hr and then placed in a cold chamber at 3°C for 18 hr. The solvent was then pressed off in a small tissue press and the alcohol was immediately taken off under vacuum at 40-50°C at a pressure of 15 mm of mercury. A preliminary experiment in which 25 lb. of thymus tissue was extracted as described above with acetone was undertaken. Upon removal of the acetone, the residue was extracted with ethyl ether and the ether soluble residue was taken up in corn oil. The aqueous residue of the remaining 100 lb. was extracted 6 to 8 times with one-half volumes of petroleum ether. The petroleum ether was removed and the residue saved for injection and for further fractionation into 70% alcohol soluble, petroleum ether soluble, and benzene soluble fractions. The aqueous residue following removal of petroleum ether soluble substances was then extracted with 1, 1, trichlorethane, boiling point 113°. The 1, 1, trichlorethane was then removed under vacuum and the residue saved for injection and further fractionation into petroleum ether and 10% alcohol soluble fractions. Ethyl ether and benzene extractions of the remaining aqueous residues were made in 3 ten-lb. lots following the procedures already described above.

Methods. Possible evidence of metabolic changes following and attributable to subcutaneous injections of thymus extracts was sought through these several experimental approaches: (1) Blood sugar determinations in the rat at 1, 3, 9 and 24 hr after injection; (2) insulin sensitivity in the mouse[3] and (3) rat, as titrated by injections of 20 U of insulin per kg per hr with serial blood sugar determinations until convulsions occurred; (4) the blood sugar reaction of the diabetic rat (animals made diabetic by partial pancreatectomy, by 14 daily injections each of 30 U of insulin per kg, and by starvation for 7 days); (5) changes in leucocyte equilibria; and (6) the relative weights and histology of the endocrine organs, liver, spleen, and kidneys following injections of extract.

Results. The effect of the thymus extracts on the blood sugar of 58 rats fed and fasted was tested. Nine experimental rats received 100 g tissue equivalents of ethyl ether soluble substances in corn oil obtained directly from aqueous residues following the removal of acetone. The 9 control animals received only corn oil. Blood sugar values in fed and fasted animals at 1, 3, 6, 9, and 24 hr after injection showed no significant variations from those observed in the controls. Twenty experimental and 20 control animals were given thymus 1, 1, trichlorethane soluble substances in tissue equivalents of 150-200 g, petroleum ether soluble substances in tissue equivalents of 150-200 g, and aqueous residues in tissue equivalents of 30-60 g. The fat soluble substances produced no significant changes in blood sugar taken 1, 3, 6 and 9 hr after injections, as compared with controls treated with similarly prepared tissue control extracts. Intraperitoneal injection of thymus aqueous residues produced blood sugars above 250 mg but similar levels were obtained with control tissue aqueous residues.

The insulin sensitivity of 120 mice[3] (60 experimental and 60 control) was tested using 100-150 g tissue equivalents of 1, 1, trichlorethane soluble substances. Other fractions of the extract were tried but found to be too toxic for use in this experiment. Thymus extracts gave a 25% protection in terms of survival time as compared with

[3] Jensen, H., and Grattan, J. F., *Am. J. Physiol.*, 1940, **128**, 270.

corn oil, but similarly prepared kidney extracts in the same tissue equivalence gave 75% protection as compared with corn oil. Ethyl ether soluble substances extracted from thymus aqueous residue after the removal of petroleum ether and 1, 1, trichlorethane soluble substances also showed some protection which was, however, equal to or less than that of the control tissues.

Rat insulin sensitivity was tested using 40 rats (20 experimental and 20 control) injected with all of the fractions noted above. None of these gave even suggestive protection. Blood sugar samples were taken hourly until the onset of convulsions.

The effect of the extracts was analyzed in a study of 15 diabetic animals. Three were made diabetic by partial pancreatectomy, 6 by injection of 30 U per kg of regular insulin daily for 2 weeks, and 6 by starvation for 7 days. Petroleum ether soluble substances were tried in the depancreatized animals and the results were negative. Trichlorethane soluble substances were tested in the remaining temporarily diabetic animals and again no significant elevation in the blood sugar was noted as compared with the controls. Blood sugar samples were taken at 1, 3, 6 and 9 hr, following injection of 200 g tissue equivalent of extract.

In studying the weights of endocrine glands, spleen, liver, and kidneys, 54 animals (27 experimental and 27 control) were injected daily for 18-21 days with 125-75 g tissue equivalents of the extracts described. Thymus weights in animals receiving the petroleum ether soluble fraction were found to be about 50% below and adrenal weights 50% above those injected with corn oil alone. No significant changes in endocrine size were noted as compared with control animals and experimental and control liver and spleen weights were similarly proportionate to total experimental and control body weights. Thymus size was readily correlated with toxicity in all extracts used, both experimental and control.

Changes in body weights were recorded in all animals injected over long periods and in no instance was any experimental group heavier than its control.

White blood counts and differential studies were made in a sampling from each group of animals and no significant differences were noted.

Discussion. Certain of the effects attributed to "thymhormon" by Bomskov may be nonspecific. For example, it has been found that destructive irradiation of the thymus produces profound cytologic and functional changes in the near-by thyroid gland[4] and to a lesser extent in the hypophysis. The resulting endocrine changes may be responsible for the claim that the thymus gland is the mediator of the pituitary diabetogenic effect. Furthermore, it should be noted that injection of any substance of sufficient toxicity may cause retrogression of secondary sex characteristics.

Recent studies by Rawson *et al.*[5] suggest that whole thymus tissue will inactivate the pituitary thyrotropic hormone, an effect not observed with tissues other than the thyroid gland itself. This may or may not bear a relation to Bomskov's observation that "thymhormon" inhibits the effect of the thyrotropic hormone.

In considering thymus tissue extracts as a whole, it is felt that the lipoid extracts described in the literature previously were too toxic to serve any physiologic function. This conclusion is suggested by the resulting thymus atrophy, adrenal hypertrophy, weight loss, and tissue reaction at the site of injection following their use. We chose an acetone and (or) alcohol, petroleum ether, trichlorethane method for extracting a great part of our tissue since it has been previously shown to be satisfactory in the making of potent and non-toxic extracts from adrenal cortex tissue.[6]

Summary. None of the metabolic functions studied have been significantly or specifically influenced by extracts of the thymus gland under the conditions cited.

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[4] Hashimoto, E. I., and Freudenberger, C. B., *J. A. M. A.*, 1939, **112**, 1680; Jolles, B., *Am. J. Roentgen.*, 1941, **45**, 259.

[5] Rawson, R. W., Sterne, G. D., and Aub, J. C., *Endoc.*, 1942, **30**, 240.

[6] Thatcher, J. S., Dept. of Physiol., Ohio State University, personal communication.

13896

Aspartic Acid as a Partial Substitute for the Growth-Stimulating Effect of Biotin on *Torula cremoris*.

STEWART A. KOSER, MARJORY H. WRIGHT AND ALBERT DORFMAN.
From the Department of Bacteriology and Parasitology, The University of Chicago.

In recent work in this laboratory with *Torula cremoris* it was found that aspartic acid partially substituted for the growth-stimulating effect of biotin. Studies on the accessory factor requirements of this yeast, which will be published in detail elsewhere, have shown that nicotinamide, pantothenic acid, thiamin, and biotin are all needed for rapid growth with development of marked turbidity in 24 hr. Omission of any one of these 4 factors produced either a slight delay in the rate of growth, a marked delay, or no appreciable growth, depending upon the factor omitted.

When biotin was omitted the results varied with the basal medium employed in the tests. In an ammonium phosphate-glucose-inorganic salt medium omission of biotin resulted in a very marked delay, visible growth appearing only after 4 to 7 days or longer, at 37°C. When a mixture of 19 amino acids was substituted for ammonium phosphate the omission of biotin produced only slight delay; uniformly good growth was produced at 48 hr, though only light growth or no growth at 24 hr.

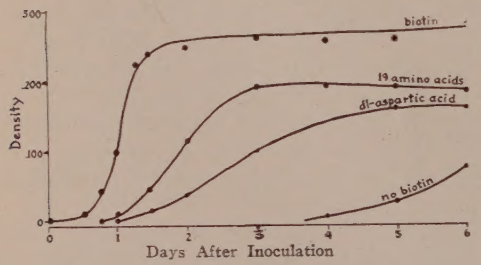

FIG. 1.

Effect of aspartic acid, amino acid mixture, and biotin on growth of *Torula cremoris* in an ammonium phosphate-glucose-inorganic salt basal medium. Nicotinamide, calcium pantothenate and thiamine were added in each instance in the amount of 0.2 μg per ml. Biotin, when present, was supplied in the amount of .002 μg per ml.

Density (L = 2-log G) was determined with the Evelyn colorimeter equipped to hold selected tubes of 15 mm diameter in place of the standard Evelyn tubes.

Incubation was at 37°C.

To determine whether this stimulation of growth in the absence of biotin could be related more definitely to certain of the amino acids, additional experiments were performed in which amino acids were supplied singly and in various combinations. It was found that much of the growth-stimulating effect of the original amino acid mixture was supplied by 1(—)-aspartic acid. Two additional samples of 1(—)-aspartic acid and one of synthetic dl-aspartic acid were tested with identical results.

A typical experiment is given in Fig. 1. In the presence of the other 3 accessories, but without biotin, growth of *T. cremoris* was quite slow, requiring in this instance 4 to 5 days before it became evident. In some experiments growth did not appear until 7 days or more after inoculation. When the amino acid mixture was supplied growth appeared much sooner, though not as promptly as in the presence of biotin. Much of the growth stimulation produced by the amino acid mixture was reproduced by either 1(—)-aspartic acid or dl-aspartic acid. Since this effect was produced by synthetic dl-aspartic acid it seems unlikely that the stimulation caused by this compound is due to the presence of biotin as an impurity.

Rather large amounts of aspartic acid were necessary to produce stimulation such as that shown in Fig. 1. Usually 100 or 200 μg per ml of medium were used, however 40 μg gave growth almost equal to 100 μg while 20 and 10 μg produced successively less stimulation.

The other 18 amino acids of our original mixture were also tried singly. With the exception of 1(+)-glutamic acid no noteworthy stimulation of growth was observed.

This amino acid produced some stimulation in the absence of biotin but it was not as marked as that caused by aspartic acid. The use of dl-glutamic acid resulted in less stimulation than that produced by the natural amino acid, so that the presence of biotin as an impurity may have been partially responsible for the results with the l(+)-compound.

Alanine, which along with aspartic and glutamic acids has been found to play a prominent role in transamination reactions,[1] produced no stimulation under the conditions of our tests.

Asparagine and glutamine produced marked stimulation but the presence of biotin as impurity appears likely and thus far no samples have been secured which could be regarded as definitely free of biotin.

In view of the recent work on the structure of biotin and the suggested relationship of pimelic acid to part of the biotin molecule[2,3] it seemed of interest to determine the effect of pimelic acid on growth of *T. cremoris* in the absence of biotin. The ammonium phosphate-glucose medium was used with nicotinamide, thiamin and calcium pantothenate. To separate tubes of this were added pimelic acid and also pimelic acid and cysteine together.[4] Experiments were performed with 10 μg and 100 μg of each compound per ml of medium.

No stimulation of growth was apparent when pimelic acid and cysteine were supplied. When these two compounds were present with dl-aspartic acid there was no appreciable stimulation over that caused by the aspartic acid itself. Thus, although *T. cremoris* grows but slowly in the absence of biotin, a supply of pimelic acid and organic sulfur does not facilitate cell multiplication in the simple basal medium used here. These results are in contrast to those secured with aspartic acid.

A possible explanation of the activity of aspartic acid might be that biotin is concerned in the formation of this amino acid and that its inclusion in the medium decreases the need for biotin. Another explanation might be that this amino acid is capable to a limited extent of performing a function of biotin. However, we have no direct evidence for either of these possibilities.

Conclusion. The omission of biotin resulted in a pronounced delay of growth of a stock laboratory strain of *Torula cremoris* in an ammonium phosphate-glucose-inorganic salt medium. Under these conditions l(—)-aspartic acid or dl-aspartic acid, when supplied in place of biotin but in much larger amounts, produced a distinct stimulation of growth. A less marked stimulation was caused by glutamic acid. Seventeen other amino acids did not produce this effect.

Pimelic acid and cysteine did not stimulate growth in the absence of biotin under the conditions of these tests.

1 Cohen, P. P., Transamination, Chapter in *A Symposium on Respiratory Enzymes,* University of Wisconsin Press, Madison, 1942.

2 Hofmann, K., Melville, D. B., and du Vigneaud, V., *J. Am. Chem. Soc.,* 1941, **63**, 3237.

3 du Vigneaud, V., Dittmer, K., Hague, E., and Long, B., *Science,* 1942, **96**, 186.

4 Eakin, R. E., and Eakin, E. A., *Science,* 1942, **96**, 187.

13897 P

Electron Micrography of the Western Strain Equine Encephalomyelitis Virus.*

D. G. Sharp, A. R. Taylor, Dorothy Beard and J. W. Beard.
From the Department of Surgery, Duke University School of Medicine, Durham, N.C.

A material behaving biologically as the Western strain equine encephalomyelitis virus has been isolated as previously noted[1] from extracts of chick embryo tissue diseased with this agent. Recent work in this laboratory has resulted in the isolation of the virus in preparations of high homogeneity as indicated by the character of sedimentation diagrams in the analytical ultracentrifuge. Among the studies made on the purified material was the examination of it by means of the electron microscope. The results obtained with this instrument are reported briefly in the present paper.

The Western strain of equine encephalomyelitis virus was cultured in 11 or 12-day-old chick embryos in a manner similar to that employed for the Eastern strain of this virus.[2] The diseased tissue was extracted in Ringer solution of pH 8.6 to 8.8 for 4 days between 5° and 8°C. The extracts, cleared by low-speed centrifugation and filtered with celite, were subjected to 1 or 2 ultracentrifugal cycles in alternate high (30,000 g) and low (17,000 g) centrifugal fields. The purified product in Ringer solution was cleared of aggregates by angle centrifugation (7000 g) for 5 to 10 min. Electron microscope preparations were made by applying to the collodion film thin layers of virus in undiluted Ringer solution or Ringer solution diluted with water to 0.03 M salt concentration. Some of the screens were allowed to dry and examined without further treatment. Others were dried and then washed with Ringer solution or with distilled water. The purified virus was examined the day of isolation and at intervals thereafter for 40 days. Parallel studies were made on changes in infectivity and sedimentation characters.

Electron micrographs of fresh virus preparations giving a sharply sedimenting boundary in the analytical ultracentrifuge showed the presence of circular images with little evidence of extraneous material. An example of the findings is given in Fig. 1 obtained 3 days after isolation of the material. The association of virus properties with such preparations and the absence of images of this sort in micrographs of healthy embryo tissue indicated the probability that the images represented electron micrographs of the virus particles.

The virus particles were consistently difficult to photograph immediately and within a few days after isolation. The images were of low contrast and their edges indefinite. This is illustrated in Fig. 1 where the micrograph of 15,700 × magnification was increased to 52,500 × by enlargement from the negative. In older preparations the contrast was greater. The images in micrographs of fresh specimens were for the most part rounded. Some were oval in appearance while others appeared definitely elongated. Of especial interest was a clearly discernible differentiation of structure within the images. This was seen in the presence of a dense inner region surrounded by an enveloping region of considerably less density. In general the denser area was rounded, centrally placed, occupied much of the image and appeared to be approximately the same density throughout. In many cases, however, the inner area appeared to be of approximately half the total diameter of the particle and was oval or irregular in outline.

* This work has been aided by a grant from Lederle Laboratories, Inc., Pearl River, New York, and by the Dorothy Beard Research Fund.

1 Sharp, D. G., Taylor, A. R., Beard, D., Finkelstein, H., and Beard, J. W., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 459.

2 Taylor, A. R., Sharp, D. G., Beard, D., and Beard, J. W., *J. Infect. Dis.*, in press.

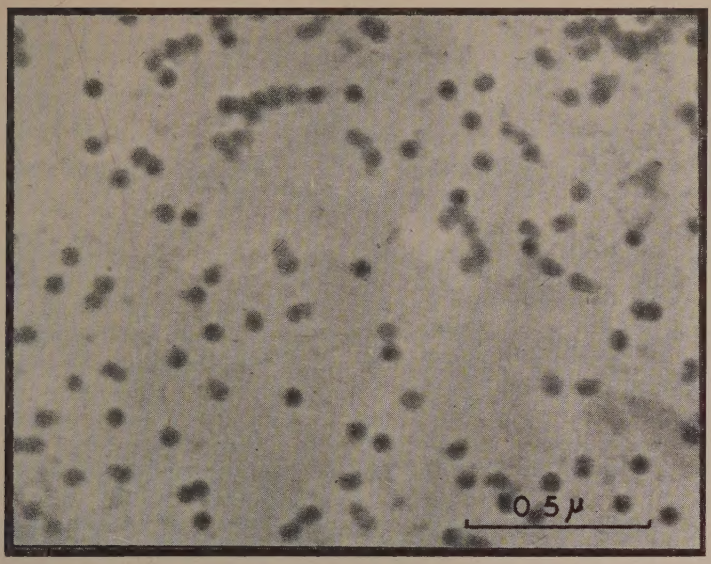

FIG. 1.

Electron micrograph of Western strain equine encephalomyelitis virus (52,500 ×) obtained 3 days after ultracentrifugal purification. The micrograph was made at 55 kilovolts. The magnification in the final pictures was calculated on the basis of measurements on tobacco mosaic virus rods which have been reported to be 15mμ in diameter.

Occasionally the inner structure appeared double. These findings were consistent whether the specimen was examined without washing on the screen or was washed with Ringer solution. Washing with distilled water markedly decreased contrast and definition of the less dense enveloping region.

The general features of the fresh preparations were seen also in the older specimens. With aging, however, the images appeared definitely to gain in contrast and to change in shape. The predominant image in preparations 3 weeks after isolation was comma-shaped, with the denser area forming the eccentrically placed head and the less dense material constituting the tail piece. The infectivity of such preparations had decreased 3 decimal dilutions but the sedimentation pattern and constant had not changed.

Measurements of diameter on 20 images in each of 4 plates of 3 different preparations gave values of 39.6, 39.3, 38.0, and 42.1 mμ, with an average value of 39.8 mμ for all. These values were calculated on the basis of comparison with the tobacco mosaic virus which has been reported[3] to be 15 mμ in diameter.

The present findings indicate that the Western strain equine encephalomyelitis virus is a spherical or disc-shaped particle of approximately 40 mμ in diameter. Electron-micrographic images reveal an internal structure characterized by a round or oval region of relatively high density surrounded by an enveloping material of less density.

[3] Stanley, W. M., and Anderson, T. F., *J. Biol. Chem.*, 1941, **139**, 325.

13898

Influence of Atropine on Atrophy of Denervated Skeletal Muscle of the Rat.

ERNST FISCHER.

From the Department of Physiology and Pharmacology, Medical College of Virginia, Richmond, Va.

According to Levine *et al.*,[1] atropine decreases considerably the rate of atrophy of a denervated skeletal muscle of the rat. Since these authors used only the weight loss of the muscle as an index for the degree of atrophy, it was desirable to find out also if atropine diminishes the rate of the loss in contractile power per weight unit of the muscle and of the diminution in its birefringence.[2]

Methods. In 3- to 5-months-old rats (from our own colony of a mixed strain), the sciatic nerve of one side was resected for 1 cm under aseptic conditions. Litter mates were arranged in pairs, one rat serving as control, the other being treated immediately with atropine. The doses employed for the main series were the same as by Levine *et al.*, namely 15 mg/100 g rat daily, divided into 3 equal doses given subcutaneously as a 1% solution in the morning, afternoon and evening until the end of the experimental period.[3] After 2 weeks, the contractile power of both gastrocnemius-solei was determined, the animals killed, the muscle weight and birefringence determined with methods employed in previous investigations.[2]

Results. Although the weight loss determined as the percentage difference between denervated and normal muscle was distinctly less for the atropine treated rats than for the controls, no significant differences were found between contractile power or birefringence of the denervated muscles of the treated rats in comparison with the denervated muscles of the control. For both the treated rats and the controls, the difference in contractile power or birefringence between denervated muscle and normal muscle were of the same magnitude as reported previously for denervation atrophy of 2 weeks' duration in normal rats.[2] However, the weight of the normal muscle of the treated rats was always considerably less than that of the control litter mates (Table I). This difference in the weight of the normal muscles of control and treated rats, is probably due to the fact that from the beginning of the atropine treatment, the treated rats develop distinct anorexia and, in consequence, develop a severe cachectic inanition. In Table I, the initial and the final weights of the treated and the control rats are tabulated with the weights of their normal and denervated gastrocnemii at the end of the experimental period. In calculating the percentage differences between treated and control rats and between their normal muscles, the small initial difference in rat weights has been adjusted. As in all types of weight loss, also in this inanition, the percentage difference between normal muscle of treated and control rats is somewhat larger than the percentage difference between the total weights of the rats. Since the normal muscles of treated rats show such a distinct inanition weight loss, a calculation of the weight loss due solely to denervation by comparing denervated with normal muscle of the same treated rat would be only permissible if in simultaneous denervation and inanition atrophy the two rates of weight loss add algebraically. For various biological reasons this is not probable, and Hines and Knowlton[4] have demonstrated that fasting after denervation produces less weight loss than

[1] Levine, R., Goodfriend, J., and Soskin, S., *Am. J. Physiol.*, 1942, **135**, 747.

[2] Fischer, E., *Am. J. Physiol.*, 1939, **127**, 605; 1940, **131**, 156; PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 277.

[3] Soskin, S., personal communication.

[4] Hines, H. M., and Knowlton, G. C., *Am. J. Physiol.*, 1935, **110**, 8.

TABLE I.

Rats treated daily with atropine. Doses in mg/100 g rat. Odd experimental numbers controls; even numbers treated rats (figures given only for one litter); m, the means for 8 rats each. Duration of denervation and treatment, 2 weeks. The figures for loss or gain designate the percentage difference between treated and control rats corrected for the small difference in initial weight.

Exp. No. and dose of atropine	Animal wt			Gastrocnemius							Theoretical atrophy inanition + denervation, %
	Initial, g	Final, g	Loss, %	Normal, g	Loss, %	Denervated, g	Atrophy, %	Loss, %	Atrophy inanition correct., %	Gain, %	
15 mg											
A 1	245	269		1.376		.800	41.9		41.9		
A 2	234	166	28.1	1.052	19.8	.696	34.8	16.9	51.6	23.2	61.7
A 3	238	245		1.572		.847	46.1		46.1		
A 4	235	184	24.9	.941	39.3	.706	25.0	45.6	54.4	18.0	85.4
m (control)	251	267		1.486		.787	47.1		47.1		
m (atrop.)	250	197	26.0	1.017	31.3	.700	31.2	33.7	52.7	11.9	78.4
10 mg											
m (control)	300	302		1.806		.938	48.1		48.1		
m (atrop.)	306	266	13.6	1.443	21.6	.822	43.1	10.4	52.2	14.7	69.7
5 mg											
m (control)	269	278		1.620		.882	45.5		45.5		
m (atrop.)	267	262	5.8	1.484	7.8	.809	45.4	0.2	49.7	9.2	53.3

the sum of the weight losses due to fasting alone and denervation alone.

From a practical standpoint, a denervation atrophy delaying drug is only of value if the weight difference between denervated muscle of the treated rat and normal muscle of the control is less than the difference between denervated and normal muscle of the control. In the next to the last vertical column of Table I this method of calculation was used after adjusting the weight of the normal muscle of the control in proportion to the initial difference in rat weights. It is obvious from these figures that atropine does not decrease, but actually increases the rate in weight loss after denervation. However, this increase is less than the algebraic sum of atropine and denervation weight loss (last column Table I). To make sure that the simulated beneficial effect of atropine is only due to the atropine inanition, in 2 further experimental series only ⅔ or ½ respectively, of the original dose of atropine was administered. The mean values for these series are found also in Table I. It is obvious that as soon as the inanition effect of atropine becomes small the simulated atrophy retarding effect of atropine disappears. This explains also why Levine *et al.* found that only extremely large doses of atropine were effective in their experiments.

Summary. Atropine does not retard the diminution in contractile power per weight unit nor the diminution in birefringence in denervation atrophy of rat muscle. The apparent retardation of weight loss of the denervated muscle in atropine-treated rats found by comparing the percentage weight difference between normal and denervated muscle of treated rats with the difference found in controls, does not indicate a true atrophy retardating effect of atropine. Continuous administration of atropine in such large doses produces cachectic inanition of the rats, in consequence of which the weight difference between denervated and so-called normal muscle is diminished, since the latter weighs considerably less than a truly normal muscle.

13899

Metabolism of Nitrogen and Acetone Bodies in Fasting Hypophysectomized Rats After Low Protein Diet.

Reginald A. Shipley.

From the Department of Medicine of Western Reserve University, School of Medicine, and the Lakeside Hospital, Cleveland, Ohio.

It has been known since the work of Voit[1] that nitrogen excretion during fasting is directly related to the protein content of the preceding diet. MacKay and his associates[2] have recently shown that in the rat the administration of a low protein diet is followed by a decreased excretion of nitrogen during fasting. The latter workers have attributed this finding to the impoverished supply of storage protein available for release. There is a question, however, whether protein may ever be regarded as an inert component of protoplasm, and it is therefore conceivable that when protein intake is restricted a mechanism for the conservation of cellular protein comes into prominence and that its influence persists during subsequent fasting. The well-known exaggeration of the ketosis of fasting after a low protein diet might depend upon the relative deficiency of anti-ketogenic substances derived from protein incident to a suppression in protein catabolism. Inasmuch as the anterior pituitary is intimately concerned with the anabolism of protein, it might be suspected that this increased ketosis and decreased nitrogen elimination are due to a persistence of pituitary hypersecretion in response to the threat of cellular protein depletion when the dietary intake of protein is low. In order to determine the part played by the pituitary in the metabolic adjustments resulting from a restricted protein intake, the response of hypophysectomized rats to fasting was observed in a group of animals receiving a low protein diet and was compared to the response of another group receiving a normal diet. For controls, 2 similar groups of unoperated rats were also observed.

Methods. All rats were of Sprague-Dawley strain and weighed between 96 and 135 g. The hypophysectomized animals were observed for stationary weight for one to 3 months before use, and the completeness of the operation was verified by serial microscopic sections. All of the unoperated control rats were males while about half of the operated animals on each diet were females. No sex difference in response was noted. Purina dog chow served as the normal diet. The low protein diet which was given for 2 to 4 weeks before fasting was composed of the following:

	%
Casein (Labco "Vitamin free")	8
Hydrogenated Cotton Seed Oil	38
Rice Starch	44
Cod Liver Oil	2
Brewers' Yeast (Anheuser-Busch Strain G)	3
Salt Mixture (McCollum and Simmonds No. 185 with added fluoride, iodide, and manganese)	5

The body weight of both operated and unoperated rats remained practically constant while this diet was being given.

After a 24-hr fast a sample of tail blood was taken from all animals and analysis for the total content of blood acetone bodies was made by a micro-method.[3] During the second 24 hr, the urine was collected in small glass metabolism chambers and the total nitrogen excretion determined. Animals that did not survive more than a day after cessation of the fast were excluded.

Results. It may be observed from Table I that like normal rats the hypophysectomized animals excreted less nitrogen and exhibited greater ketonemia after a low protein diet than after a normal diet. It is therefore

1 Voit, C., *Z. f. Biol.*, 1866, **2**, 307.

2 MacKay, E. M., Carne, H. O., Wick, A. N., and Visscher, F. E., *J. Biol. Chem.*, 1941, **141**, 889.

3 Shipley, R. A., and Long, C. N. H., *Biochem. J.*, 1938, **32**, 2242.

TABLE I.
Blood Acetone Body Levels and Excretion of Nitrogen.

	No. of rats	Blood acetone bodies (mg%)	No. of rats	Nitrogen excr. mg/100 g—24 hr	% loss body weight—48 hr
Normal Rats					
Dog Chow Diet	18	10.8 ± 1.5*	9	124 ± 5.8	23 ± 0.66
Low Protein "	17	17.5 ± 2.6	13	84 ± 3.4	11 ± 0.43
Hypophysectomized Rats					
Dog Chow Diet	29	4.6† ± 0.40	15	93 ± 3.5	12 ± 0.32
Low Protein "	28	10.1‡ ± 0.85	11	73 ± 4.6	8 ± 0.40

*Standard error of the mean.
†Avg level of 7 of these rats before fasting 0.4 ± 0.04.
‡Avg level of 9 of the group before fasting 1.0 ± 0.5.

evident that these metabolic responses to protein restriction are not entirely dependent upon the hypophysis but must depend at least in part upon adjustments within tissues elsewhere. It may be noted that the hypophysectomized animals on both diets showed less ketonemia than the corresponding control rats. This cannot be due to increased protein catabolism inasmuch as the nitrogen excretion was lower in the operated animals. The decrease could be due, however, to the absence of the hypophyseal ketogenic principle. The presence of a definite ketosis after hypophysectomy indicates that during fasting the ketogenic principle of the pituitary is not indispensable to the operation of the ketogenic mechanism as a whole. Oastler and Anderson[4] have observed that a high degree of ketosis can be induced by fat feeding in the hypophysectomized rat.

Weight loss differed significantly among the several groups during fasting. The hypophysectomized rats lost less weight than did the animals in the unoperated groups. This is undoubtedly attributable to the low basal metabolism which follows hypophysectomy. The relatively small weight loss of both normal and hypophysectomized animals after a low protein diet is striking. Protein oxidation is decreased in these animals and fat oxidation presumably is high. The greater energy which is supplied by the oxidation of fat in preference to protein could account in part for the comparatively slight decline in body weight. Substitution of an equicaloric weight of fatty tissue for muscle would require the consumption of only one-tenth the mass of body substance. The decreased weight loss after a low protein diet may also be due in part to the decreased metabolism which has been observed in rats receiving a diet of this nature.[5]

Conclusions. Hypophysectomy does not prevent the decrease in nitrogen excretion or increase in ketosis during fasting after a low protein diet. These responses therefore have their origin at least in part in other tissues.

The technical assistance of Miss Ethel Buchwald is gratefully acknowledged.

[4] Oastler, E. G., and Anderson, A. B., *Biochem. J.*, 1939, **33**, 1094.

[5] Horst, K., Mendel, L. B., and Benedict, F. G., *J. Nutrition*, 1934, **8**, 139.

13900

Changes in Adrenal Function During the Alarm Reaction.

S. Albert. (Introduced by Hans Selye.)

From the Department of Anatomy, McGill University, Montreal, Canada.

Previous investigators have shown that during the alarm reaction produced by nocuous stimuli the adrenal cortex hypertrophies[1–3] and that this enlargement is accompanied by a definite hyperfunction.[4] Weil and Browne[5,6] demonstrated that, when the human organism is subjected to stress, the excretion of corticoids in the urine is significantly increased above the normal. Davidson,[7] Howard,[8] and Burrill and Greene[9] demonstrated the production of testoids by the adrenals of young castrate male rats and mice. Recently Forbes[10] has shown that the characteristic reaction of the adrenal to stress results first in an increase (for 12-48 hr) then a decrease in 17-ketosteroid excretion.

It seemed of interest to demonstrate whether the enlarged adrenal produces excessive amounts of testoid or folliculoid compounds during the alarm reaction. The weights of the seminal vesicles and the prostate were used as indicators of testoid activity in the castrate male, while the vaginal smear was used as an indicator of folliculoid activity in the spayed female.

Twelve mature male albino rats were castrated and divided into 2 equal groups; one group received 0.3 cc of a 10% solution of formalin subcutaneously thrice daily for 12 days and the other served as non-injected controls. On the thirteenth day all animals were killed. The adrenal glands, seminal vesicles and prostate were weighed after fixation in Heidenhain's "Susa" mixture. In the non-injected control group the adrenals averaged 36 mg (range: 34-38 mg), the seminal vesicles 93 mg (range: 80-114 mg), the preputial glands 32 mg (range: 23-39 mg) and the prostatic complex 94 mg (range: 68-133 mg). In the formalin-treated animals the adrenals weighed 54 mg (range: 46-63 mg), the seminal vesicles 87 mg (range: 81-94 mg), the preputials 29 mg (range: 20-36 mg) and the prostatic complex 94 mg (range: 83-111 mg).

A similar experiment was performed on spayed mature albino female rats. Four days after gonadectomy injections were started. Daily vaginal smears were taken during the entire experiment to determine whether estrus would reappear. The control adrenals averaged 41 mg (range 38-47 mg) and the preputial glands 45 mg (range: 31-55 mg), while in the formalin-treated animals the adrenal weighed 56 mg (range: 42-64 mg) and the preputials 38 mg (range: 29-48 mg). At no time during the entire experiment did estrus appear in any of the animals.

These experiments demonstrate quite clearly that in rats, during the alarm reaction produced by the injection of a solution of 10% formalin, there is a marked increase in the weight of the adrenal glands, but no significant change in that of the accessory sex organs or of the vaginal smear. We may, therefore, conclude in agreement with Forbes[10] that during exposure to stress the stimulation of adrenal function is such as to give maximum protection against stress, resulting in a specific overproduction of

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1 Selye, Hans, *Endocrinology*, 1937, **21**, 169.

2 Selye, Hans, *Can. Med. Assn. J.*, 1936, **34**, 706.

3 Freud, J., Manus, M. B. C., and Mühlbock, O., *Acta brev. Neerl.*, 1938, **8**, 6.

4 Dosne, C., and Dalton, A. J., *Anat. Rec.*, 1941, **80**, 211.

5 Weil, Paul, and Browne, J. S. L., *Science*, 1939, **90**, 445.

6 Weil, Paul, and Browne, J. S. L., *J. Clin. Invest.*, 1940, **19**, 772.

7 Davidson, C. S., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 703.

8 Howard, Evelyn, *Proc. Am. Physiol. Soc.*, 1938, p. 105.

9 Burrill, M. W., and Greene, R. R., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 327.

10 Forbes, A. T., Preliminary report published by the Josiah Macy, Jr., Foundation, 1942.

corticoids without a corresponding increase in either testoids or folliculoids.

Summary. In gonadectomized animals treated with 10% formalin solution there occurred a marked hypertrophy of the adrenal glands, but no significant change in the size of the accessory sex organs (vaginal smear and preputial glands in the female; seminal vesicles and prostate in the male). It is concluded that the adrenal cortical hypertrophy, so characteristic of the alarm reaction, is not accompanied by increased sex hormone production.

The cost of this investigation was defrayed by a grant from the Blanche E. Hutchinson Fund of McGill University administered by Professor Hans Selye.

13901

Effects of Anoxia at Birth on Central Nervous System of the Guinea Pig.

W. F. WINDLE* AND R. F. BECKER

From the Anatomical Laboratories and the Institute of Neurology, Northwestern University Medical School.

Studies on intrauterine respiration, respiratory changes at birth and tolerance by the newly born of anoxia have been reviewed elsewhere.[1] It has been observed that manifestations or cerebral pathology and impairment of the intellect are frequently associated with histories of cyanosis and apnea at birth.[2,3] Some investigators have cautioned that nerve cells of the young may be even more susceptible to damage by anoxia than those of the adult. These and similar clinical studies have been questioned because they were based on selected cases, and fully adequate controls were not available.[4] In the present experiments in guinea pigs with litter-mate controls, the thesis that asphyxia neonatorum can elicit irreparable destructive changes in the central nervous system has been tested.

The abdomen of the animal at or near term was anesthetized with 1 cc of a 1% solution of procain hydrochloride. No other anesthetic was employed. One fetus was delivered immediately through a midline incision to serve as a control. The uterine vessels were then clamped or the umbilical cords of the remaining fetuses were compressed through small incisions in the uterus.

Varying degrees of anoxia were induced in 103 animals. Fifty-eight of them were delivered just before or just after intrauterine respiratory movements, induced by the anoxia, had ceased. Survivors (38%) were those which suffered no anoxial apnea and required no resuscitation. Forty-five guinea pigs were delivered after 6 to 21 min of anoxia, after the fetal heart had become slow and often after it could no longer be palpated. Resuscitation was accomplished in 71% of them by inflating the lungs with oxygen or oxygen containing 10% carbon dioxide. The gases were administered from small rubber bags attached to hypodermic needles which were inserted into the trachea. Respiration was simulated by compressing and releasing the trachea above the needle. Resuscitation was accomplished in a few minutes to more than an hour; the time required was not consistently related to the duration of anoxia. These animals exhibited characteristic symptoms of asphyxia pallida; namely, apnea, atonia, bradycardia, relaxation of anal sphincters, and cold pale skin.

Results. All experimental animals, including those in which anoxial apnea had not

* Aided by grants from the Women's Faculty Club of Northwestern University Medical School and from the Clara A. Abbott Fund.

1 Windle, W. F., *Ann. Rev. Physiol.*, 1943, **5**, in press.

2 Schreiber, F., *J. A. M. A.*, 1938, **111**, 1263.

3 Clifford, S. H., *J. Pediat.*, 1941, **18**, 567.

4 Galaway, C. E., in discussion of Schreiber's paper.[2]

been reached, exhibited symptoms of central nervous system damage lasting from a few hours to more than a month. All showed motor weakness and tremors, were unable to get onto their feet during the first hour or more after respiration had been established, and when they did right themselves, usually held the head to one side tending to fall in that direction. They rarely cried and did not respond to bright flashes of light or loud sounds. In contrast, the 90 controls were alert, cried and righted themselves in a few minutes although many were slightly tremorous and weak for a few minutes to an hour.

Pronounced and persistent symptoms resulted in 40 animals. Partial flaccid paralysis was more commonly observed in the hind than in the forelimbs. In one instance, a picture of complete spinal transection was seen. Decerebrate states were observed in many animals after respiration had been established. The spasticity was more marked in the fore- than in the hind limbs and was sometimes accompanied by opisthotonus. Recovery was more rapid than in cases of flaccid paralysis and was usually complete within 24 hr. In some animals it lasted more than a week. Persistent tremors and fortuitous convulsive movements were commonly encountered. The facial as well as the limb muscles were involved in some animals. Rhythmical running movements often occurred in the early phase of recovering from anoxia. Incoördination of muscular effort was observed in nearly all experiments. Ataxia frequently persisted for several days and occasionally persisted a week or longer. Recovery from motor impairment was highly variable, but usually seemed to be complete in a few days. Some animals remained partially paralyzed until they died or were killed a week to 10 days after birth. Most of them showed no evidence of motor disturbances after two weeks.

Sensory functions appeared to be affected in varying degree after anoxia at birth. During the early hours after resuscitation, many animals were hyperirritable. Touching the nose, limbs or body often led to convulsions or fits of running movements. Irritability usually subsided in an hour or 2 and by the second day the guinea pigs exhibited impairment of sensation. They were less responsive to tactile and painful stimuli than their normal litter-mates and some appeared to have lost cutaneous sensations to a marked degree. Most animals lost the startle response to sharp sounds.

Somnolence was observed in some of the experiments. The animals which had been anoxemic closed their eyes and lay quietly in the cage for many minutes at a time during the first one to 3 days. They would go to sleep while standing, the head would drop, and they would topple over before awakening. Reduced activity levels were encountered even 3 to 4 weeks after birth when animals were tested in a simple maze.

The behavior of all experimental and control animals was observed daily from birth until they died or were killed 1 day to 8 weeks later. Even after motor symptoms were no longer evident it was possible to observe behavior differences by critically comparing the animals which had been asphyxiated with their litter-mates. The experimental guinea pigs were less irritable, more docile and less aware of changes in the environment than the controls. The experimental animals were undisturbed by new situations, *e.g.*, a maze or problem box, in contrast to their litter-mates which often showed marked frustration.

The brains of all specimens were preserved and are being studied histologically. Great variation in histological changes have been encountered in 17 specimens compared with their normal litter-mates. Histopathology was questionable or slight in 6 animals subjected to less than 8 min of anoxia. Definite, often marked, changes were present in all others. Generalized necrosis of brain and spinal cord with chromatolysis, edema, small hemorrhages and ventricular enlargement appeared during the period from 2 to 5 days after birth. Glial proliferation and loss of nerve cells, especially in pyramidal layers of the cerebral cortex, was observed between 5 and 9 days; and generalized atrophy together with glial scars marked the older specimens (14-43 days). The most severe pathology

was not encountered in those specimens subjected to the longest periods of anoxia. The sites of damage varied widely from experiment to experiment.

Comparison of the present results with those in adult animals[5,6] reveals points of difference which are attributable to characteristics of the young animal. The nerve cells of the newly born appear to be much less vulnerable than those of the adult and withstand anoxia for greater lengths of time. There seems to be less specificity in respect to anoxial damage in the newborn than in the adult.[7]

[5] Weinberger, L. H., Gibbon, M. H., and Gibbon, J. H., Jr., *Arch. Neur. and Psychiat.*, 1940, **43**, 961.

[6] Thorner, M. W., and Levy, F. H., *J. A. M. A.*, 1940, **115**, 1595.

[7] Kabat, H., and Grenell, R. S., *Anat. Rec.*, 1942, **82**, Sup. p. 33.

Summary. Anoxia induced at birth invariably produced symptoms of neural damage. Transient shock, tremors, ataxia, and incoördination were not usually associated with impaired behavior or brain pathology. More than 8 min of anoxia lead to such symptoms as decerebrate states, marked ataxia, convulsions, paralysis, hyper- and hypoesthesia and somnolence, correlated in some cases with behavioral changes. Highly variable nonspecific devastation of brain and cord tissues, occasional hemorrhages, gliosis and generalized atrophy were encountered.

All experiments were controlled with healthy litter-mates and permit the conclusion that neonatal asphyxia (asphyxia pallida) may induce irreparable fortuitous destruction of large or small regions of the brain.

13902 P

Response to Murine Poliomyelitis Virus (Lansing Strain) of Mice on Different Levels of Thiamin Intake.*

CLAIRE FOSTER, JAMES H. JONES, WERNER HENLE AND FRIEDA DORFMAN.

From the Departments of Pediatrics and Physiological Chemistry, School of Medicine, University of Pennsylvania, and the Children's Hospital of Philadelphia.

The following is a preliminary report on the relation of thiamin intake to the response of mice inoculated with the Lansing strain of murine poliomyelitis virus.[1] This investigation is part of a long-range program for the study of the relation of diet to resistance to infection.

In each of the 4 experiments summarized here, litters varying from 21 to 31 days of age were split among the groups being compared. Each mouse (except one uninjected group in Experiment I) was injected intracerebrally with 0.03 ml of a 0.5% suspension of mouse brain in saline. Normal mouse brain was used for the controls. Mouse brain infected with the Lansing strain of mouse-adapted poliomyelitis virus served as the virus inoculum. This amount of virus corresponded to between 10 and 100 fifty-percent-mortality doses.

Previous experience had shown that 100 μg of thiamin per 100 g of diet (called diet 100) cover the needs of the growing mouse and that 10 μg per 100 g of diet (called diet 10) lead to signs of deficiency within 15 days.

The first 3 experiments, totaling 646 mice, failed to show any significant difference between mortality of groups on diet 100 inoculated with virus and those on diet 10 inoculated with virus, injected with normal brain, or uninjected. The incidence of paralysis, on the other hand, was markedly greater in the high-thiamin than in the low-thiamin groups inoculated with virus.

Averaging the 3 experiments (each of

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

[1] Armstrong, C., *Pub. Health Rep.*, 1939, **54**, 2302.

which was carried 21 days after inoculation), mice which were placed on diet 10 for 15 to 20 days before inoculation with the virus and were continued on it, showed 13% paralysis compared with 74% in the mice on diet 100 for the same period of time. No paralysis was observed in mice injected with normal brain.

The fourth experiment was designed to determine whether or not the thiamin-deficient mice would develop paralysis if the majority of them were prevented from dying of deficiency for at least 21 days after inoculation by increasing the thiamin to a maintenance level.

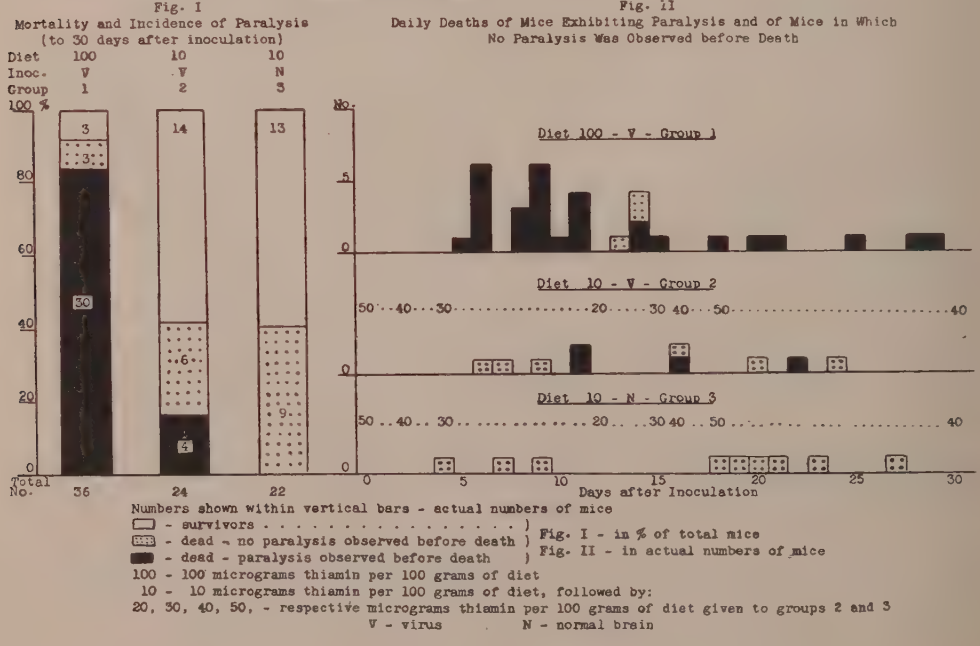
When the majority of mice on diet 10 became markedly deficient and a few of the smallest ones had died (after 26 days on diet 10), the thiamin in the diet of mice in Groups 2 and 3 (Fig. I and II) was raised from 10 to 30 μg per 100 g of diet. Thereafter, the amount of thiamin given to Groups 2 and 3 was varied in an attempt to keep the majority of these mice alive and just maintain them at approximately constant weight.

All mice were injected 34 days after institution of the experimental diets either with virus (Groups 1 and 2) or with normal brain (Group 3). There was practically no difference in mortality between Group 2 (low-thiamin diet, inoculated with virus) and Group 3 (low-thiamin diet, injected with normal brain). In contrast, the death rate was considerably higher in Group 1 (high-thiamin diet, inoculated with virus). Still more striking was the fact that the incidence of paralysis in mice on the high-thiamin diet (Group 1) was several times that in mice on the diet in which the thiamin was deficient (Group 2). Paralysis was followed by death within a few days. None of the mice injected with normal brain (Group 3) exhibited paralysis.

No explanation is offered at this time for the results observed, nor has the specificity of this reaction yet been investigated. The survival of the virus and the possible histological lesions in the thiamin-deficient mice are being studied in the current experiments.

We are grateful to Drs. J. Harold Austin and Joseph Stokes, Jr., for assistance in planning the experiments; and to James E. Benton and Mabel E. Quinby for assistance with preparation of diets and care of the mice.

SUMMARY OF EXPERIMENT IV



13903

Determination of Diodrast in Whole Blood.*

MILTON E. BOBEY AND J. WAIDE PRICE. (Introduced by J. T. Wearn.)

From the Department of Medicine, Western Reserve University, and Lakeside Hospital, Cleveland, Ohio.

In most kidney function studies involving the use of diodrast (D), this substance is determined in terms of its iodine content (I) in plasma and in urine. In some cases, however, analysis of D in whole blood is desirable and, aside from tedious ashing procedures, the only method proposed for the determination of blood D is that of White and Rolf[1] which requires a large correction factor (about 50% of the I determined) and which should be evaluated for each experiment.

The method described by Alpert[2] gives complete recovery from 1:15 zinc hydroxide filtrates of plasma containing 1-4 mg of I per 100 cc but was not applied to whole blood analyses. Direct use of this plasma method with whole blood yields a filtrate containing on the average only about 85% of the D present in the blood. The recovery may be increased but not to 100% by the use of greater dilutions, up to 1:100, in the preparation of whole blood filtrates, but this either reduces the accuracy of the analysis by lowering the amount of I in each determination or else necessitates a modification of the procedure to the determination of smaller amounts of I which largely nullifies the chief advantage of the method, its simplicity. It has been observed, however, that the recovery is inversely proportional to the cell volume as determined by hematocrit and the use of a simple recovery factor based on this observation gives the true whole blood I value within the range of accuracy of the method as applied to plasma.

The correction factor necessary to the use of Alpert's method with whole blood was obtained as follows:

Procedure. Portions of oxalated dog blood were mixed with plasma or cells from the same animal to vary the cell volume. To these bloods D, dissolved either in normal saline solution or in plasma was added so that the final cell volume ranged from 15.8 to 48.5% and the I level was equivalent to 2-20 mg %. The samples then stood for one hour at 38°C with occasional shaking to allow diffusion of D into the cells. White[3] has shown that this diffusion takes place slowly (presumably at room temperature). At 38°, however, 40 min suffices for attainment of a diffusion equilibrium between cells and plasma of the order observed in arterial blood *in vivo*. The preparation of filtrates from these bloods, digestion and titration were carried out exactly as described by Alpert. Hematocrit determinations were also done. From the known amount of D present in each sample, the percentage recovery could be calculated and correlated with the cell volume. The results of 24 determinations in 5 experiments are shown graphically in Fig. 1. The straight line fitted to the points by the method of least squares is given by the equation:

$$\% \text{ Recovery} = 99.8 - 40.0 \text{ Vc}$$

where Vc is the cell fraction as determined by hematocrit. The line extrapolates to 99.8% recovery when Vc = O (plasma) and to 59.8% recovery when Vc = 1.00 (cells). The validity of the latter figure has not been tested since the precipitating reagents do not completely remove the cell protein when employed in the proportions used for whole blood and plasma. The 99.8% recovery indicated for plasma has been verified experimentally and is in agreement with the re-

* Supported by a grant from the Commonwealth Fund.

1 White, H. L., and Rolf, D., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 1.

2 Alpert, L. K., *Bull. Johns Hopkins Hosp.*, 1941, **68**, 522.

3 White, H. L., *Am. J. Physiol.*, 1940, **130**, 654.

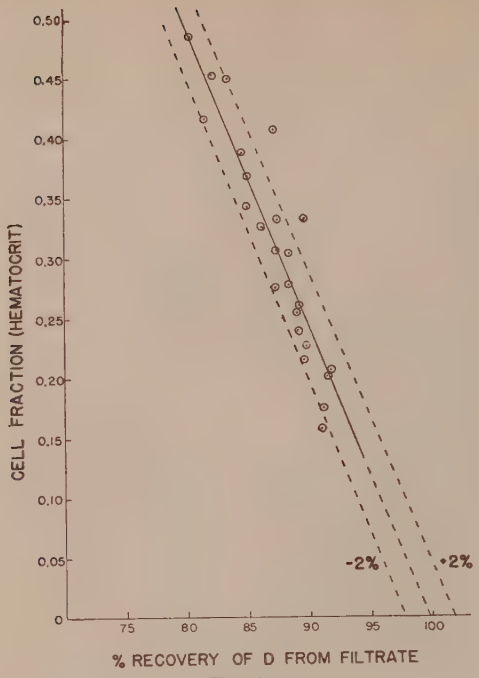


FIG. 1.

The relation between the per cent recovery of D from 1:15 whole blood filtrates and the cell fraction as determined by hematocrit. The broken lines enclose the area representing a deviation of ±2% from the theoretical value given by the equation discussed in the text.

coveries reported by Alpert for plasma levels of 1-4 mg per 100 cc. When higher levels were used in this work, up to 20 mg per 100 cc, the filtrates were diluted to be equivalent to the lower range and the recoveries were equally good. Of the 24 determinations, one deviates from the average line by +4.4%, one by +3.7%, one by —2.7%, and the remaining 21 are within ±2%.

The true whole blood I value then becomes:

$$\frac{\text{I determined in whole blood filtrate}}{99.8 - 40.0\ Vc} \times 100$$

Discussion. The correction factor discussed here applies only to dog blood. In man, the D content of cells in equilibrium with plasma is only about half of the D content of dog cells in similar equilibrium and the rate of diffusion from plasma to cells is lower.[4] Whether or not these differences would significantly change the correction factor should be determined before applying the correction to human blood or to blood of any other species. In the case of the dog, however, the recovery of D from the laked blood is dependent only upon the cell volume, regardless of whether the D was present originally in plasma alone, cells alone or was in equilibrium between the two. This presumably indicates that the recovery of D in the filtrate is dependent on the total protein present in the laked blood solution at the time of precipitation rather than on the location of the D at the time of laking, the recovery being essentially complete in the presence of plasma protein but decreasing with increase in the amount of cell protein. This influence of protein may be involved in the incomplete recoveries obtained from plasma, whole blood and cells in the method of White and Rolf.

Conclusion. Alpert's method for the determination of D in plasma may be applied to the determination of D in whole blood of dogs by the use of a simple correction factor based on the hematocrit determination of cell volume.

[4] White, H. L., Findley, T., Jr., and Edwards, J. C., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 11.

13904 P

Normal Development and Experimental Treatment of the Opossum Mammary Gland Primordium.*

DOROTHY WELLS PLAGGE. (Introduced by Carl R. Moore.)

From Hull Zoological Laboratory, The University of Chicago.

The mammary primordia may be clearly seen in serial sections of pouch areas from 1 day opossum pouch young *(Didelphys virginiana)*. The primordia become visible to the naked eye at about 12 days and may be affected experimentally with female sex hormones by the 18th day and with male hormones by the 30th day.

Normal Development. The development of the normal mammary gland from 1 day to 100 days was studied in 18 females, 11 males, and 3 animals too young to be sexed. It was found to be similar to that described by Bresslau[1] for *Didelphys marsupialis*. At one day the mammary primordium in *D. virginiana* is visible under low power magnification as a small, oval thickening of the epidermis extending into the underlying derma and surrounded by concentric layers of cells from this stratum. The oval thickening enlarges and extends deeper into the corium and then assumes a typical flask shape with the neck of the flask attached to

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[1] Bresslau, Ernst, *Z. f. Morphol. und Anthropol.*, 1902, **4**, 261.

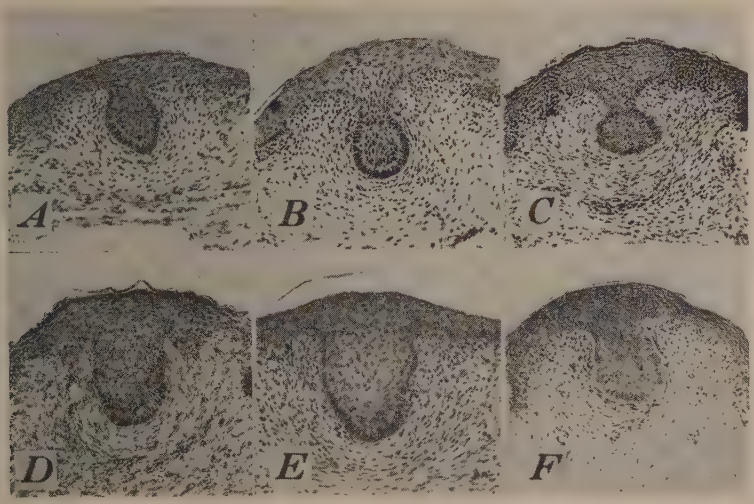


FIG. 1.
Mammary Primordia of Opossum Pouch Young, × 99.
A. 32-day normal male.
B. 32-day normal female.
C. 32-day female, testosterone propionate ointment days 16-31.
D. 30-day male, estradiol ointment days 6-29.
E. 30-day female, estradiol ointment days 6-29.
F. 32-day female, testosterone ointment days 3-31.

TABLE I.
Effects of Sex Hormones on Immature Opossum Mammary Gland.

No. of Animals	Sex	Age at death (days)	Treatment (days prior to autopsy)	Mammary gland response
			Estrogens.*	
5	F	18-24	12-18	Hyperplasia, Abnormal forms.
1	M	30	24	" " "
2	F	30-31	10-24	" " " Indication of precocious branching
3	F	35-46	29-40	" " " Definite precocious branching
			Androgens†	
2	F	16-25	11-13	Normal size. Normal forms
5	F	30-41	16-32	" " Abnormal forms. No branching
1	F	45	42	Taller than normal. Abnormal forms. No branching
1	F	62	22‡	Abnormal forms. Branches smaller than normal

*Treated with estradiol ointment.
†Treated with testosterone ointment, testosterone propionate ointment, testosterone propionate injections or combinations of the three.
‡Treated for 22 days followed by 31 days without treatment.

the epidermis, the round body extending into the underlying mesenchyme (Fig. 1,B). This primordium shows the first signs of branching in the 49-day female and by 83 days the primary milk hairs, milk ducts and sebaceous glands are present.

The development of the mammary primordium in the normal male follows the same general lines as that of the female although the embryonic male mammary gland seems somewhat smaller and less developed than that of the female in most of the stages compared (Fig. 1,A and B).

Experimental Treatment. After daily treatment with estradiol in ointment form all the mammary primordia of 10 females and one male between the ages of 18 and 46 days showed stimulation. These treated primordia were hyperplastic, of abnormal shape and exhibited precocious branching (Table I). A 30-day male (Fig. 1,D) included in this series showed a response like that of the treated female of the same age (Fig. 1,E) except for the absence of premature branching.

The response of the female mammary primordium to androgenic treatment was not nearly as striking as in animals treated with estradiol. Nine female pouch young were preserved at ages between 16 and 62 days after treatment with testosterone and testosterone propionate in ointment form and subcutaneous injections of t-propionate in sesame oil. A 16-day and a 25-day female showed little if any mammary stimulation. The primordia of the older animals, however, all responded by development of abnormal shapes (Fig. 1, C and F),

Equine gonadotropin (1 or 5 R. U. daily of gonadogen or gonadin) when injected from 7 to 23 days before autopsy did not affect the mammary primordia of 5 females between the ages of 17 and 100 days, and one male of 30 days. In these same animals, Moore[2] found only slight evidence of testicular response in the male and no effects on the ovaries in the females.

Summary. A study was made of the normal development of the mammary gland in the male and female opossum up to the age of 100 days. It was found possible to modify the normal growth pattern of the mammary primordia by treatment with estradiol, testosterone and testosterone propionate. Gonadotropic substances at the ages and dosages used did not affect the development of the glands.

2 Moore, Carl R., *Physiol. Zool.*, 1941, **14**, 1.

13905 P

Tuberculin Tests in Guinea Pigs With Purified Protein Derivative.

EMIL BOGEN.

From the Olive View Sanatorium, Olive View, California.

Increased purification and the avoidance of denaturization of an antigen would appear to have obvious advantages. Accordingly the purified protein derivative, PPD, has been recommended unreservedly to replace the crude concentration of heterogeneous substances in glycerol broth cultures of the tubercle bacillus, introduced by Koch more than half a century ago under the name of Old Tuberculin. The discovery of the occurrence of non-specific reactions with large doses of PPD[1] seemed only to indicate that smaller doses of this potent material be used. A report that PPD did not give as good reactions in the skin of guinea pigs as did O.T. seemed to be attributable to the low doses of PPD used in that study.[2]

TABLE I.
Reactions in Different Tuberculous Guinea Pigs.

	Negative	Weak	Strong	Died
PPD	2	19	72	21
O.T.	0	4	31	0
Reactions in Uninfected Controls.				
PPD	40	8	0	0
O.T.	15	1	0	0
Reactions on Opposite Sides of the Same Tuberculous Guinea Pigs.				
PPD (right side)	2	10	24 }	14
O.T. (left side)	1	7	28 }	

In June, 1941, Dr. F. B. Seibert kindly donated a generous amount of her new potent preparation of PPD[3] for further study. Comparison of this material with a commercial Old Tuberculin (Cutter) has yielded results which are quite disconcerting.

In general, the local reaction to PPD was less distinct and characteristic than that to O.T. In known tuberculous animals, the injection of 0.1 mg of PPD, which according to Dr. Seibert corresponds to from 10 to 20 mg O.T., resulted only in 2 or 3 plus reactions in the skin, with erythema and edema of varying extent, disappearing in 3 or 4 days, without the persisting necrosis or ulceration characteristic of the reaction to 5 mg O.T.

The systemic or lethal effect of the PPD, however, was much greater than that of the O.T. Although death from 5 mg O.T. in tuberculous animals is exceedingly rare, the injection of PPD in doses as low as .005 mg which correspond to less than a milligram of O.T. resulted in death of some of the animals. Altogether, about 20% of the animals given less than 0.1 mg PPD died within the following 48 hr.

Further experiments suggested that the greater systemic effect with lessened local reactivity to PPD may be due to the greater solubility and faster absorption and dissemination of the purified material. Thus the addition to the PPD of lampblack, tannic acid, or various oils, to keep it longer *in situ*, increased the local effects.

Large doses of PPD, which give reactions in a larger proportion of tuberculous animals, also gave more non-specific reactions with non-infected animals. The same was true of PPD in oil. Some of these animals had received repeated injections of the PPD, and part of the non-specific responses may have resulted from active sensitization by the earlier doses. Such sensitization has not been observed, however, following repeated use of diagnostic doses of O.T.

This is not the only instance in which purity has not been an unqualified asset in connection with a biological test or agent. The addition of alum to diphtheria toxoid, of protamine to insulin, or of oil to epinephrin, to enhance their effects, are familiar examples. A peptone suspension of a pure

[1] Furculow, M. L., Hewell, B., Nelsen, W. E., and Palmer, C. E., *Pub. Health Rep.*, 1941, **56**, 1082.

[2] Medlar, E. M., Sasano, K. T., Caldwell, D. W., and Needham, E. L., *Am. Rev. Tuberc.*, 1941, **43**, 534.

[3] Seibert, F. B., and Glenn, J. T., *Am. Rev. Tuberc.*, 1941, **44**, 9.

culture of diphtheria bacilli has been reported to give skin reactions in non-immunized guinea pigs such as are characteristically elicited by field cultures, when pure cultures suspended in water or saline does not.[4]

The particular preparation of PPD used in these experiments had been produced with less heating and denaturing than previously described products. This may account for the more consistent results on skin testing,[5] the failure to elicit therapeutic focal reactions in man[6] and the lessened antigenicity reported elsewhere.[7]

There seems to be a distinct place for PPD in testing for the occurrence of tuberculous infection. In rabbits and monkeys[8] it is possible to elicit tuberculin reactions with 1 mg PPD or more, where such reactions may not be obtained with O.T. In guinea pigs,[9] however, O.T. seems to be more sensitive, more specific, less antigenic and less often lethal, as well as less expensive, than the PPD used here.

[4] Stone, R. V., and Weigel, C., *Am. J. Pub. Health*, 1929, **19**, 1133.

[5] Seibert, F. B., *Am. Rev. Tuberc.*, Supp., 1935, **30**, 707.

[6] Thompson, B. C., *Tubercle*, 1936, **18**, 27.

[7] Seibert, F. B., *Am. Rev. Tuberc.*, 1941, **44**, 1.

[8] Schroeder, C. R., *Zoologica*, 1938, **21**, 397.

[9] Negley, J. C., and Bogen, E., *J. Urology*, 1940, **44**, 860.

13906

Fate of Mannide Monoöleate in the Animal Body.

WILLIAM E. EVANS, JR., HENRY WOLLENWEBER, MARJORIE RUPPERSBERGER AND JOHN C. KRANTZ, JR.

From the Department of Pharmacology, School of Medicine, University of Maryland, Baltimore, Md.

Owing to the shortage of glycerin in England toward the close of the world war, fats were prepared without this polyhydric alcohol. Halliburton *et al.*[1] studied the effect of mannitol oleic acid ester as a substitute for butter fat in a balanced ration for rats and observed that it possessed the same nutritional value as butter fat and prolonged feeding did not produce toxic symptoms. In the present war, glycerin is again a product of great importance. Having been interested for many years in the metabolic pattern of the sugar alcohols, we turned our attention to the fate of their fatty acid esters in the animal body. The first compound studied was mannide monoöleate.

Physical and Chemical Characteristics. Mannide monoöleate* is a viscid oily liquid with a yellow color. Its solubilities in various common solvents are similar to those of edible fixed oils and its odor is characteristic, resembling that of vegetable fixed oils.

Although this substance is referred to as mannide monoöleate it is actually a mixture composed chiefly of the monoöleic acid esters of the mannitol dianhydrides or mannide together with some minor amounts of mannitol monoanhydride monoöleates, some dioleate esters, and a little free acid. The material used had a hydroxyl number of 120, an acid number of 6.6 and a saponification number of about 165. The mannide fraction has been identified as the 1,4:3,6 dianhydromannitol or isomannide containing some 1,4 mannitan.

Single Dose Administration. Young male white rats were given 2 cc per 100 g of mannide monoöleate by stomach tube. Of the 5 animals receiving the compound none showed symptoms of distress or depression. After 3 weeks no abnormalities were found. This dosage was administered intraperi-

[1] Halliburton, W. D., *et al.*, *Biochem. J.*, 1919, **13**, 301.

* Generous supplies of mannide monoöleate were furnished by the Atlas Powder Company of Wilmington, Delaware.

TABLE I.
Influence of Mannide Mono Feeding on Fecal Fat of Rats, 10 Animals.

Day of Exp.	Diet, Purina chow plus	Food consumed, g/3 days	Fecal wt, g/3 days	Free fatty acids, %	Fecal fat (as fatty acids) Neutral fat, %	Soap fat, %	Total fecal fat, %
1	5% cotton seed oil			1.10	1.11	0.56	2.77
2		432	209	0.61	0.54	0.52	1.67
3				0.49	0.76	1.56	2.81
6	5% mannide monoöleate			0.21	0.10	1.49	1.80
7		434	188	0.92	1.49	0.01	2.42
8				0.49	0.64	1.02	2.15

toneally to 5 additional rats; they showed no symptoms other than abdominal distention, but upon autopsy there was a chemical fibrinous and fibrous type of peritonitis.

Feeding Studies (Rats). Young white male rats, 50 to 80 g in groups of 30, were fed a diet of purina chow as a control basal diet. To this were added 3 and 5% quantities of mannide monoöleate respectively. Body weight of control and experimental animals was determined weekly. At the end of 8 weeks, 10 animals in each group were sacrificed and autopsied. The growth curves of the experimental and control animals were identical within experimental error. On gross examination there were no significant abnormalities. Histological sections of the kidneys, liver, spleen and intestinal mucosa were normal.

Excretion Experiments. Over a period of 2 weeks, 8 rats were fed a diet containing 5% of mannide monoöleate. The urine of these animals upon evaporation yielded a syrupy residue, which was extracted with absolute alcohol. Upon evaporation, the alcohol yielded a large crop of crystals, which, by melting point, were proved to be isomannide. No mannitol, mannide or mannitan was found. Previous work has shown[2,3] that isomannide, mannide and mannitan are not utilized, although this ester of mannide is absorbed and hydrolyzed as shown by the appearance of isomannide in the urine.

Feeding Experiments (Monkeys). Five *Rhesus macacus* monkeys were fed 2 cc daily of mannide monoöleate for a period of 8 weeks in addition to a basal diet of apples, carrots, potatoes and sunflower seeds. Two additional monkeys were fed the basal diet. The weights of the animals were determined weekly. The feeding of mannide monooleate produced no abnormalities; there was no significant change in body weight, no diarrhœa and no pathological change in the livers or kidneys, nor were the hemoglobin index or blood smears significantly affected.

Absorption of Mannide Monoöleate. Young white rats were housed in wire-bottom cages and fed a diet of purina chow plus 5% of cotton seed oil for several days. Their fecal fat was determined daily after 2 days feeding. They were then fed purina chow containing 5% mannide monoöleate and their fecal fat again determined after 2 days feeding. The results (Table I) indicate that neither neutral fat nor total fecal fat was significantly changed by the addition of mannide monoöleate to a basal diet of purina chow instead of 5% cotton seed oil. We consider this and the excretion studies to be evidence that mannide monoöleate is absorbed from the intestinal tract of the white rat and is not reëxcreted into the gut.

Summary. In the quantities administered in these experiments mannide monoöleate appears to be neither acutely nor chronically toxic to the white rat and to be innocuous in the diet of the *Rhesus macacus* monkey. Mannide monoöleate is absorbed from the intestinal tract of the white rat. No damage was observed to the important viscera of the white rat or monkey after an 8-week feeding period with mannide monoöleate.

[2] Carr, C. J., Musser, R., Schmidt, J. E., and Krantz, J. C., Jr., *J. Biol. Chem.*, 1933, **102**, 721.

[3] Krantz, J. C., Jr., Evans, W. E., Jr., and Carr, C. J., *Quart. J. Pharm. and Pharmacol.*, 1935, **8**, 213.

13907

Response of Focus of Origin of Experimental Ventricular Extrasystoles to Warming or Cooling.

David Scherf. (Introduced by Israel S. Kleiner.)

From the Department of Medicine, New York Medical College, Flower and Fifth Avenue Hospitals.

Extrasystoles show the phenomenon of coupling, that is, in a given individual there is a constant interval between the extrasystole and the preceding beat. This is accepted as proof of a close relationship between them, the preceding beat releasing the extrasystole in some way.

According to one group of observers the releasing beat, after having spread over the heart, reënters a small path in a circumscribed area which has been traversed before, causing a second response. Others believe that in a small "island" within the heart conduction is possible in one direction only (unidirectional block). The stimulus enters the island only on one side and on leaving this area after some delay it excites neighboring fibers causing an extrasystole. According to a third hypothesis the releasing beat initiates an abnormal, heterogenetic stimulus formation in some center.

A reëntry mechanism, caused by unidirectional block has been demonstrated in experiments on muscle strips[1] and, under rare conditions, in the mammalian heart *in situ*.[2] However there is little to support the assumption that this mechanism prevails in the formation of the majority of the extrasystoles daily encountered in patients.

Piccione and Scherf[3] demonstrated that the application of hypertonic solutions of sodium chloride or barium chloride on the epicardium or subepicardially is followed by paroxysmal tachycardias and extrasystolic arrhythmias similar to the various clinical types. The extrasystoles show fixed coupling. A specific ion effect is involved.

The effect of cooling or warming the area of the exposed heart upon which the salt solution was applied is reported in this paper. The influence on the resulting arrhythmia received particular attention. Warming or cooling was accomplished by sending water with a temperature of 150°-175°F or 50°-60°F respectively through a copper thermode. The apex of the thermode had a diameter of 4 mm. The experiments were performed on 26 dogs under nembutal anesthesia (0.5 cc per kg) with artificial respiration.

On heating the focus of origin the number of extrasystoles, already present, was regularly increased so that tachycardias appeared instead of coupled beats; if a tachycardia was present, its rate increased. When extrasystoles with fixed coupling or tachycardias failed to appear after the application of salt solution, they developed if heat were applied to this area. On cooling the focus, the rate of a tachycardia, when present, slowed and extrasystoles were abolished; however, they reappeared following an interval of about 40 sec after the cooling was stopped.

The first tracing in Fig. 1 (4,13,1942) shows a paroxysmal ventricular tachycardia, which appeared after the subepicardial injection of 0.1 cc of a 10% solution of sodium chloride to the region of the conus of the right ventricle. The rate of the tachycardia was 187. Warming the injected area with the thermode increased the rate to 214. The white irregular perpendicular lines indicate the beginning of warming. On interrupting this the original rate returned, but rewarming the area again increased the rate.

The second tracing in Fig. 1 (1,13,1941) shows a trigeminal rhythm which appeared after injecting 0.1 cc of a 30% solution of sodium chloride into a circumscribed area of the conus of the right ventricle. The dis-

[1] Schmitt, F. O., and Erlanger, J., *Am. J. Physiol.*, 1921, **87**, 326.

[2] Scherf, David, *Arch. Int. Med.*, 1941, **67**, 372.

[3] Piccione, F. V., and Scherf, David, *Bull. N. Y. Med. College, Flower and Fifth Ave. Hosps.*, 1940, **3**, 83.

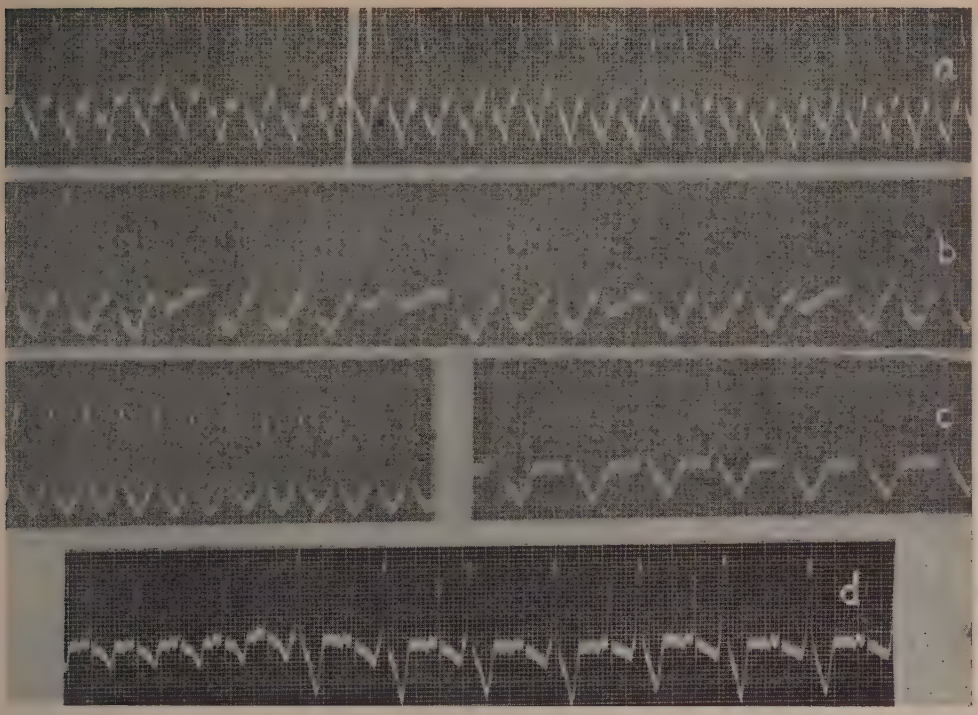


FIG. 1.

Fig. 1a shows a ventricular tachycardia following subepicardial injection of sodium chloride; the rate increases during application of heat to the injected area.

Fig. 1b shows a trigeminal rhythm following subepicardial injection of sodium chloride; the first part of Fig. 1c shows the increase in rate during warming, the second part the decrease in rate during cooling the injected area.

Fig. 1d shows a bigeminal rhythm which appeared exclusively and repeatedly during warming an area of the conus of the right ventricle on which sodium chloride was injected subepicardially.

tance between two extrasystoles was constant, 0.36 second (rate: 166 per min). After warming the area of injection a paroxysmal tachycardia appeared (first part of the third tracing in Fig. 1) and the distance between two extrasystoles fell to 0.26 second (rate: 230), during cooling (second part of the third tracing) the rate fell to 115. A few seconds later the extrasystoles disappeared completely.

The fourth tracing in Fig. 1 was obtained in an experiment on 5,14,1942. This time the subepicardial injection of 0.1 cc of a 10% solution of sodium chloride into the conus area of the right ventricle failed to produce extrasystoles. After warming the area where the injection had been given, short runs of extrasystoles in groups and bigeminal rhythm appeared. The fourth tracing in Fig. 1 shows a regular bigeminy. Each extrasystole follows the normal beat after an interval of 0.24 second. The extrasystoles appeared repeatedly 2 or 3 seconds after warming was begun and disappeared 4 to 6 seconds after the removal of the thermode.

Warming areas on the surface of the heart to which no sodium or barium chloride had been applied, did not evoke extrasystoles.

It is difficult to conceive that warming the area of injection accelerates an existing circus or reëntry mechanism, or even initiates them, if they were not previously present. These results seem to be explained better by the assumption that the extrasystoles are due to a disturbance of stimulus formation. If the

latter is due to formation and discharge of potential differences across a semipermeable membrane, this process may be changed depending on the presence of certain electrolytes and it may be accelerated or even initiated on warming and slowed down or abolished by cooling the center of extrasystoles formation.

Conclusions. Coupled extrasystoles appear in dogs following epicardial or subepicardial application of hypertonic solutions of sodium or barium chloride on circumscribed areas. Warming of the injected area increases the number and the rate of the extrasystoles or causes them to appear even if they were not present after the injection. Cooling the area of injection slows the rate of formation of extrasystoles or abolishes them completely.

These results can be explained satisfactorily by assuming a heterogenetic stimulus formation in the treated area.

13908 P

Inhibiting Effect of Methionine, Choline and Betaine on Rabbit's Susceptibility to Infection with Vaccinia.*

DOUGLAS H. SPRUNT.

From the Department of Pathology, Duke University School of Medicine, Durham, N.C.

In the course of experiments to determine what factors in a rabbit's diet are essential to virus multiplication it was observed that the subcutaneous injection of methionine increased the rabbit's resistance to dermal infection with vaccinia. Six experiments have been performed with methionine. The results of a typical experiment are shown in Table I. In this experiment and others described below rabbits weighing approximately 2 kg were used. The experimental rabbits received 300 mg of methionine subcutaneously daily for 2 days preceding and 5 days following vaccination. In other experiments the conditions of the experiment were varied as to the amount of methionine and the times at which it was given. These experiments showed that if the methionine was given 48 hr before vaccination, the resistance was increased 14 fold; if given immediately following vaccination, the increase was 10 fold; if delayed until 48 hr after vaccination the increase was 5 fold. In addition to these quantitative changes in resistance, the lesions of the animals whose resistance had been stimulated most by methionine were smaller and disappeared approximately 24 hr earlier than did the ones of the control animals. Measurements of resistance were made by use of serial dilutions of vaccinia virus as described previously.[1] The experimental error in this method is considerably less than a 2-fold difference, hence the observed changes were highly significant.

Additional studies were then undertaken to determine what biological functional group of the methionine molecule was responsible for this effect. That it was probably not dependent on the generalized properties of the amino acids as such was indicated by the fact that glycine had no significant effect on susceptibility. Neither does the mere presence of sulfur in the molecule explain the action, for cystine did not duplicate the effect of methionine.

The chemical characteristic which seems most likely to be involved is the biologically labile methyl group. Choline and betaine share with methionine the property under proper circumstances of possessing an easily transferable methyl group,[2] and these substances were therefore investigated in a series of 5 experiments. When given 48 hr before

* Aided in part by Duke University Research Council.

1 Sprunt, D. H., *J. Exp. Med.*, 1942, **75**, 297.

2 du Vigneaud, V., *Biological Symposia*, 1941, **5**, 234.

TABLE I.
Effect of Methionine, Choline and Betaine on Susceptibility of Rabbit to Infection with Vaccinia as Indicated by the 50% Point.

Chemical used		No. of rabbits used	50% point log	Inc. resistance ×
	Controls	5	5.65	
Methionine	Exper.	5	4.40	18
	Controls	6	6.00	
Betaine	Exper.	6	5.28	5
	Controls	6	5.40	
Choline	Exper.	5	4.12	19

and continued until 5 days after vaccination, betaine showed little effect on the lesions for the first 2 days following inoculation of the virus; after this initial lag period, however, its action became manifest and resulted ultimately in a 5-fold increase in resistance. There was, however, considerable variation between rabbits, some showing little change from the controls and others a marked decrease in the number and size of the lesions. The results of a typical experiment with betaine are shown in Table I. The experimental rabbits were injected subcutaneously with 500 mg of betaine hydrochloride which was neutralized with soda bicarbonate. These injections were made at approximately 8-hr intervals. They were begun 48 hr before and continued for 5 days after vaccination. Choline also caused an increase in resistance (20-fold). The results of a typical experiment are shown in Table I. In this experiment 300 mg of choline chloride was given to the experimental rabbits at same intervals as the betaine in the above experiment. The lesions in the animal receiving the choline remained small throughout the experiment. The effect of the choline, however, cannot be attributed definitely to a specific action for the animals treated with this substance did not eat as well as the controls and lost some weight. It is therefore conceivable but unlikely that the consequent increase in resistance may have been due to loss in weight for fasting has been shown previously to decrease susceptibility to vaccinia infection.[1] The degree of weight loss was not nearly so great in the rabbits receiving the choline (5%) as in those which were purposely fasted (18%) and in which increased resistance occurred.

The results obtained with 2 substances and to a less extent with a third containing biologically labile methyl group—methionine, choline and betaine—show that these substances inhibit dermal infection with vaccinia in the rabbits. Further investigations of this possibility are now under way. If the observed effects are proven for other viruses and other animals, it is possible that a chemotherapeutic agent effective against viral infections may be discovered among these or related chemical substances.

13909

Storage of Manganese by Thyroid. Effect on Oxygen Consumption of the Guinea Pig.

T. W. Ray and L. J. Deysach. (Introduced by P. F. Swindle.)

From the Departments of Biochemistry and Physiology, Marquette University School of Medicine, Milwaukee.

Levine and Sohm[1] observed hyperactivity of rats following the feeding of small amounts of manganese chloride. A similar observation was made by Ray[2] on mice. Sato,[3] working with rabbits, found that the injection of small doses of manganese chloride caused an increase in oxygen consumption whereas large amounts greatly decreased oxygen consumption. These observations suggest that small doses cause hyperfunction of the thyroid gland.

In order to determine whether the increased activity in animals following manganese administration actually results from the element accumulating in the thyroid, guinea pigs were injected subcutaneously with solutions of manganese chloride of different concentrations daily for 6 days and oxygen consumption values were obtained before and after the manganese injections. The manganese content of the thyroid gland as well as of other organs, was determined by the procedure of Ray.[4] Forty-eight guinea pigs were used in these experiments. All of these experimental animals received a basal diet consisting of 10 parts of rolled oats, 1 part of bran, 0.1 g of sodium chloride, cod-liver oil and cabbage.

It was earlier observed by Ray[2] that when mice were allowed to drink water containing 0.05 g of manganese chloride per liter, the element readily accumulated in the liver as well as in other tissues of the body. The liver of the normal mouse was found to contain about 3 times as much manganese per unit of weight as the remainder of the body. However, the concentration in the body per unit of weight equaled that in the liver after the salt was given in the drinking water for 15 days. In 40 days both the body and the liver became completely saturated (1.2 mg of manganese per 100 g of dry tissue). No greater amount could be found in the tissues of animals after 50, 80, or 90 days of manganese administration. When manganese chloride was injected subcutaneously into the guinea pig, however, the element was stored in quantities much greater than that found in mice receiving the salt orally. Table I shows that after injection all tissues of the guinea pig examined stored manganese in varying amounts. The larger the dose injected, the greater was the amount stored. The thyroid appears to be peculiar in that normally it contains relatively little manganese, but it has the property of storing it in amounts out of all proportion to that stored by other organs. It may also be seen from Table I that the dog thyroid exhibits the same property of storing manganese as that of the guinea pig. When manganese chloride was injected subcutaneously into the dog in a dosage of 5.0 mg per kg body weight, the thyroid stored 49 times the amount found in the normal gland. Guinea pigs receiving a like amount of manganese chloride per kg stored 50 times the normal amount.

The data presented in Table II show that when manganese chloride was injected subcutaneously into the guinea pig in dosages ranging from 1.0 mg per kg body weight to 10.0 mg per kg body weight, the amount of oxygen utilized by the animal was markedly reduced (—10.91% to —31.4%). On the other hand, as is also shown in this table, when the small amount of 0.01 mg per kg of manganese chloride was injected, the oxygen consumption was augmented (+8.4%). It is of interest to note that the oxygen con-

1 Levine, Victor E., and Sohm, Herbert A., *J. Biol. Chem.*, 1924, **59**, Proc. Am. Soc. Biol. Chem., xlviii.

2 Ray, T. W., unpublished data.

3 Sato, Juniti, *Arch. int. de Pharmacodyn. et de Therapi*, 1929, **36**, 49.

4 Ray, T. W., *J. Biol. Chem.*, 1940, **134**, 677.

TABLE I.
Mg Mn per 100 g Dry Material After Subcutaneous Injection of $MnCl_2$ for 6 Consecutive Days.

Daily injection $MnCl_2$ per kilo guinea pig	mg 0.0	mg 1.0	mg 2.5	mg 3.5	mg 5.0	mg 10.0	mg 15.0
Liver	1.16*	1.20	1.40		1.90	6.00	13.00
Testes	0.40	0.41	0.48		0.83	0.69	1.25
Pancreas	0.92	1.60	3.69		—	—	4.86
Salivary gl.	0.75	1.75	2.40		4.05	2.95	4.95
Thyroid	?†	3.91	6.94		9.33	12.50	26.50
Daily inj. $MnCl_2$/k dog	0.0			3.5	5.0		
Liver	0.84			1.80	2.30		
Thyroid	0.17			4.00	8.52		

*Each figure following an organ in the table is the average for the manganese in mg per 100 g of dried tissue for not less than 5 animals.

†A total of 23 thyroids, 5 from the human being, 6 from the horse, and 4 each from the cow, the calf, and the dog were analyzed. The average of these was 0.19 mg manganese per 100 g of dry thyroid. Since the amount of manganese in the thyroid of the normal guinea pig is too small for accurate quantitative determination, 0.19 mg was assumed to be the average for the normal animal.

sumption values became progressively less as the storage of manganese increased in the thyroid.

Summary. 1. Subcutaneous injection of manganese chloride caused a storage of manganese in all parts of the body but especially in the thyroid gland. 2. Injection of small doses (*e.g.* 0.01 mg per kg) of this salt produced an increase in the oxygen consumption of the guinea pig, while large amounts (*e.g.* 10 mg per kg) reduced the oxygen consumption in this animal. 3. Oxygen consumption values in the guinea pig became progressively less as the storage of manganese increased in the thyroid.

TABLE II.
Effect of Manganese Chloride on Oxygen Consumption of Guinea Pigs.*

Dose $MnCl_2$ per kg, mg	O_2 used control, cc/kg/hr	O_2 after $MnCl_2$, cc/kg/hr	% deviation from control	Mg Mn recovered per 100 g dried thyroid
10	631.41	432.61	—31.4	12.50
5	634.2	535.28	—15.5	9.33
2.5	507.54	442.93	—12.73	6.94
1.0	532.73	474.6	—10.91	3.91
.01	671.0	727.93	+ 8.4	not determined

*All of the oxygen consumption results were obtained by a special metabolism apparatus devised by L. J. Deysach.

13910

Comparative Study of Effects of Testosterone Propionate Administered Intraperitoneally and Subcutaneously.

H. S. Rubinstein.

From the Laboratory for Neuroendocrine Research, Surgical Division, Sinai Hospital, Baltimore, Md.

It is now generally recognized that the potency of hormones may vary in accordance with their mode of administration. Working with the female rat, Israel, Meranze, and Johnson,[1] for example, have observed that the liver counteracted the effect of estrogens, a fact which they have used to explain the lessened potency of this hormone when administered intraperitoneally.

Working with the androgens, Deanesly and Parkes[2] also concluded that intraperitoneal injection resulted in minimal effectiveness. They, however, felt that this was due to the rapid absorption which the peritoneal lining favors.

In a previous communication[3] observations concerning the efficacy of intraperitoneally administered testosterone propionate were reported.

The following study offers added evidence that the potency of testosterone propionate is not necessarily diminished when administered intraperitoneally, but under certain conditions may even be enhanced.

For this study 45 male albino rats *(Mus norvegicus var. albus)* of Wistar Institute Strain and of approximately similar body weights were divided into 3 groups, each containing 15 respective litter mate brothers. Each animal in Groups 1 and 2 received 50 γ testosterone propionate (Perandren) dissolved in 0.1 cc sesame oil daily for 10 days. In Group 1, this was given intraperitoneally; in Group 2, subcutaneously. Animals in Group 3 served as litter mate controls. The first injection was given at 22 days of age; the last at 31 days, 24 hr after which all animals were weighed and sacrificed. Seminal vesicles (empty) and testes (without epididymides) were dissected and weighed.

The data were statistically analyzed and observed differences were considered to be "probably significant" only if the significance ratios were 3 or more.[4]

Results. From Table 1 it may be seen that body weights before and after treatment were statistically within the limits of random sampling for all groups.

The seminal vesicles became significantly larger by 274% for those intraperitoneally treated, and by 185% for those subcutaneously treated.

The testes of both treated groups were significantly smaller than those of the controls. For the animals treated intraperitoneally this was —41.9%, while for the subcutaneously injected animals it was —52.8%.

Discussion. Although when statistically considered, differences in body weights between the groups remained within the limits of random sampling, it is interesting that the group treated intraperitoneally did show a final body weight which was 7.53% less than that of the controls. This decrease at the end of treatment when added to the 3.86% by which the same group was heavier (than their controls) prior to treatment shows a total weight change of 11%. When this total weight change (*i.e.* gain in weight) is submitted to statistical methods it discloses a significance ratio of 2.56. While this, too, fails to reach the criterion set for "probable significance," its magnitude certainly indicates a trend: namely, that of inhibition of body growth (*i.e.* weight) by testosterone propionate. Since it has been shown by more protracted experiments that

1 Israel, S. L., Meranze, D. R., and Johnson, C. G., *Am. J. Med. Sci.*, 1937, **194**, 835.

2 Deanesly, R., and Parkes, A. S., *Proc. Roy. Soc.*, 1937-38, **124**, 279.

3 Rubinstein, H. S., and Kurland, A. A., *Endocrinology*, 1941, **28**, 495.

4 Pearl, R., *Medical Biometry and Statistics*, 2nd edition, Saunders, Philadelphia, 1930.

TABLE I.
Effects of Androgen Administration to Rats.*

		Difference from control		
		Wt	%	S.R.
Initial Body Wt (g)				
Control	31.1 ± 0.4			
Intraperitoneal test	32.3 ± 0.7	+1.2 ± 0.81	+3.86	1.48
Subcutaneous test	32.1 ± 0.6	+1.0 ± 0.72	+3.24	1.39
Final Body Wt (g)				
Control	63.8 ± 1.6			
Intraperitoneal test	59.0 ± 1.5	—4.8 ± 2.19	—7.53	2.19
Subcutaneous test	64.8 ± 1.9	+1.0 ± 2.50	+1.57	0.40
Seminal Vesicular Wt (mg)				
Control	16.6 ± 1.0			
Intraperitoneal test	62.1 ± 3.9	+45.5 ± 4.1	+274	11.10
Subcutaneous test	47.3 ± 3.2	+30.7 ± 3.3	+185	9.31
Testicular Wt (mg)				
Control	556.2 ± 21.7			
Intraperitoneal test	323.6 ± 18.9	—232.6 ± 28.8	—41.9	8.10
Subcutaneous test	262.1 ± 16.3	—294.1 ± 25.0	—52.8	11.75

*A comparison of body weights, seminal vesicular and testicular weights of animals injected with 50 γ testosterone propionate daily for 10 days (from 21 to 31 days of age) either intraperitoneally or subcutaneously with their litter brother controls. S.R. indicates "significance ratio."

suitably small doses stimulate[5,6] and large doses inhibit body growth,[7] it must be inferred that in all probability the 50 γ dosage was more potent (for this sized animal) by intraperitoneal injections than by subcutaneous administration.

This difference in potency is also reflected in the effect on the seminal vesicles, a comparison of which (Table I) shows how much heavier (1.47 ×) those of the intraperitoneally-injected animals grew than those of their subcutaneously-injected brothers. This finding substantiates the earlier observation[3] made with 20 γ dosage which also resulted in larger seminal vesicles (by 1.22 ×) in intraperitoneally-injected animals than in their subcutaneously-injected controls.

The effect of testosterone propionate on testicular weight is also different for the two methods of administration. Here, however, the depressing effect is greater by subcutaneous injection (—52%) than by intraperitoneal administration (—41.9%). This response differs from that previously reported for 20 γ experiments. With the smaller dosage, the testicular depression was more marked by intraperitoneal treatment than by subcutaneous injections. As a matter of fact, the effect may be said to be quantitatively diametrically opposite. For example, with 20 γ dosage intraperitoneally, testes were depressed 26%; with 20 γ subcutaneously, testes were depressed 20%. This shows a 1.3 × greater depression by intraperitoneal than by subcutaneous injection. A similar comparison made for our 50 γ experiments shows a 1.26 × greater depression by subcutaneous than by intraperitoneal injection.

On the basis of our earlier experiments it was felt, with conservatism, that the effect of testosterone propionate was approximately the same whether the hormone was injected intraperitoneally or subcutaneously. The confirmation yielded by our 50 γ experiments as shown by the seminal vesicular response indicates that for some tissues the effects produced by intraperitoneal injections may be even more striking than those obtained subcutaneously.

[5] Rubinstein, H. S., and Solomon, M. L., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 442.

[6] Shay, H., Gershon-Cohen, J., Paschkis, K., and Fels, S. S., *Endocrinology*, 1941, **28**, 877.

[7] Rubinstein, H. S., Jurland, A. A., and Goodwin, M., *Endocrinology*, 1939, **25**, 724.

There seems to be no doubt, therefore, that for experiments such as described testosterone propionate can be more potent for some tissues by the intraperitoneal route than when given subcutaneously. Interestingly enough, when similar doses are used for longer periods of time (*e.g.* from 26 to 80 days) the seminal vesicle may respond better to subcutaneous than to intraperitoneal injections.[8]

Summary. A comparative study of the potency of testosterone propionate administered intraperitoneally and subcutaneously in 45 respective litter mate male albino rats has been made. Injections of 50 γ daily dosage were given for 10 days from 22 to 32 days of age. Body weights before and after the experiment, final seminal vesicular- and testicular-weights were statistically compared. It was found that the intraperitoneally-injected animals gained 11% less weight, and showed a 274% *increase* in seminal vesicular growth, and a 41.9% *decrease* in testicular size over their controls. The subcutaneously injected animals showed practically no difference in gain in weight, a 185% *increase* in seminal vesicular weight, and a 52.8% *decrease* in testicular size compared to their controls. The differences observed for seminal vesicular and testicular weights were statistically "probably significant." The diminished weight increase observed for the intraperitoneally-injected animals while not statistically significant was sufficiently large to indicate a trend.

It is concluded that under the conditions of the experiments cited, testosterone propionate may vary in potency in accordance with its method of administration. But generalizations are as yet invalid since the greater testicular effect (depression) was observed by subcutaneous injection, and the greatest seminal vesicular response (stimulation) resulted from intraperitoneal administration.

The author acknowledges with appreciation the aid rendered by the Ciba Pharmaceutical Products, Inc., for helping defray the expense and for supplying the Perandren used in this study.

[8] Rubinstein, H. S., unpublished data.

13911

A Study of Clot Firmness in Viscometer Tubes.

JOSEPH J. LALICH* AND ALFRED L. COPLEY. (Introduced by C. J. Weber.)

From the Hixon Laboratory for Medical Research, University of Kansas, School of Medicine, Kansas City, Kansas.

Determinations of clot firmness have been made by studying the tensile strength of clots. Fonio[1] prepared plasma clots with magnesium sulfate solution and ether. He pressed the clots into discs, suspended them, and determined their tensile strength. Kristenson[2] who criticized this method used clots from plasma which was prepared by centrifugation in ice jackets. Following incubation at 37° C. for 1 hour he wrapped both ends of the coagulum in linen cloth and tied a string around each end. He then measured the tensile strength by adding weights until the clot tore. Schnedorf[3] permitted blood to coagulate around two wire meshes which served as points of attachment. Five to 30 minutes after coagulation at room temperature, weights were added to measure the tensile strength. By employing different amounts of blood and using several modifications of the same principle, he was unable to obtain comparable results. To test clot firm-

* George A. Breon Fellow in Experimental Medicine.

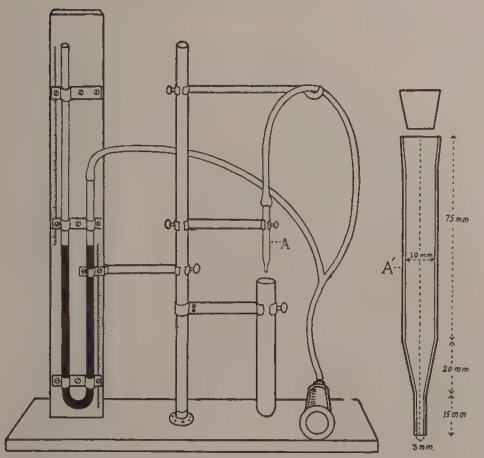
[1] Fonio, A., *Schweiz. med. Wchnschr.*, 1921, **11**, 146.

[2] Kristenson, A., *Acta med. Scandinav.*, 1932, **77**, 351.

[3] Schnedorf, J. G., personal communication, 1942.

ness we constructed a viscometer tube which measures the flow properties of blood clots. Results obtained in this way were sufficiently uniform and exhibited a measurable difference in clot firmness.

CLOT FIRMNESS APPARATUS.



Method. Glass tubes (A'; Fig. 1) are constructed with a wide portion of 10 mm and an outlet 3 mm in internal diameter. The axial length of the tube is 11 cm, its outlet being 1.5 cm, its conus portion 2 cm and the wide part 7.5 cm long. A "00" rubber stopper is fitted into the top, the tube is inverted and filled with 5 cc of blood through the outlet. Admixture of air bubbles with the blood must be prevented. This is very important, since we found that mixing air bubbles with the blood may change the firmness of the clot significantly. Tissue juice which enters the syringe initially can affect the firmness of the clot. For this reason, when 15 cc of blood were drawn, 2 tubes were filled and the remaining 5 cc were discarded. The coagulation time is determined by slightly tipping the tube at intervals of 30 sec. After the blood has coagulated, the tube is incubated at 37°C for 30 min. The viscometer tube is inverted while the stopper is removed, and the retracted clot and the serum are allowed to settle against the outlet. The tube is placed into a clamp with the outlet directly above a vessel that catches the tested material (Fig. 1). The viscometer tube is then connected to a vasolined 50 cc syringe and to a U tube mercury manometer capable of measuring 450 mm of Hg pressure. Pressure is slowly increased with the syringe by increments of 10 mm of Hg until the clot is forced out of the viscometer tube at a rate, if possible, of 5 mm per min. The pressure necessary to produce this rate of flow is taken as the index of firmness of the clot. Blood was drawn from the antebrachial vein of humans, the heart of rabbits, and the jugular and femoral veins of dogs.

Results. Similar tubes of 8 and 10 mm in internal diameter correspondingly required

TABLE I.
Agreement of Clot Firmness of Blood Withdrawn on Different Days from the Same Animal.

No. of blood withdrawals	Clot firmness in mm of Hg pressure						
	Dog I	II	III	IV	Rabbit I	II	III
1	130	170	160	420	450	320	440
	100	170	140	400	440	300	440
2	100	210	160	360	410	300	380
	100	210	150	370	380	320	320
3	100	230	140	350		460	420
	100	200	120	340		260	360
4	140	210	130	250		280	
	120	210	130	400		290	
5	190	240	140	270			
	120	200	160	300			
6	130	210	130				
	140	200	160				

TABLE II.
Coagulation Time and Clot Firmness of Blood from One Hemophiliac.

May 5, 1942			May 11, 1942		
Tube	Coagulation time, min	Clot firmness, mm of Hg	Tube	Coagulation time, min	Clot firmness, mm of Hg
1	48	50	1	91	10
2	16	110	2	85	0
1*	88	15	3	91	0
2*	88	10	4	91	0
3*	78	0	5	94	10
4*	23	90	6	33	30

*Blood from the second withdrawal.

different pressures to force the clots through an outlet, 3 mm in internal diameter. Clots from the same dog were incubated in 8 mm and 10 mm tubes for 30 min. Pressures of 30 to 40 mm of Hg were needed in 12 different 8 mm tubes, while 12 different 10 mm tubes required pressures of 100 to 140 mm of Hg to force the clots out.

The effect of incubation on clot firmness was studied in 3 normal dogs and 1 normal rabbit. Six 10 mm tubes were filled from the same withdrawal in each case. Duplicate determinations of clot firmness were done after 30, 60, and 120 min. The findings showed that a period of 30 min suffices to produce maximal clot firmness, and that incubation of 2 hr did not further alter the clot firmness.

Table I demonstrates that duplicate tests done on clots from the same animal on different days are reproducible. In a few instances the values do not agree closely, however, agreement can be obtained by repeating the test. It may be noted that dog IV whose clots were firmer than those of the 3 normal dogs was operated 3 weeks previously. Blood clots of normal rabbits constantly exhibited greater firmness than those obtained from normal dogs or humans. In a group of 8 normal humans clot firmness varied from 80 to 160 mm of Hg. Results from duplicate tests in this group agreed within 30 mm of Hg.

Table II shows the coagulation time and clot firmness in blood obtained from one hemophiliac. The clots were incubated from 30 to 40 minutes at 37° C. On the first day during the first withdrawal 10 cc of blood were drawn with difficulty. The blood which was emptied last from the syringe had the shortest coagulation time and a firm clot. On the second withdrawal 20 cc of blood were obtained. In this instance blood emptied last from the syringe also had the shortest coagulation time. Clot firmness in the last tube was within the range found with clots from normal subjects, while the clots in the remaining 3 tubes were soft. Six days later 30 cc of blood were drawn without difficulty. At this time the last tube showed the shortest coagulation time and the greatest clot firmness, even though all clots were soft. No pressure was necessary for 3 clots, whereas in 3 other clots only minimal pressure was required.

Comment. We realize that this clot firmness test is empirical. For this reason the viscometer tubes have to be standardized to obtain comparable results. By this means, one determines the applied stress at which the blood clot begins to flow. Beyond this yield value,[4] which determines the firmness of the clot, we did not study the flow properties of blood clots. Clot firmness may vary from the same withdrawal especially when the coagulation time is prolonged and the blood is mixed with tissue juice. When 5 cc of blood which entered the syringe first were discarded, the difference in duplicate tests became minimal. We found that clots from normal animals required 30 min incubation at 37° C to attain their maximal firmness. Incubation from 30 to 120 min did not produce significant changes in the firmness of blood clots from normal animals. After the blood

[4] Copley, A. L., Krehma, L. C., and Whitney, M. E., *J. Gen. Physiol.*, submitted.

gelates completely, incubation for 30 min is necessary, since at temperatures of 20 to 25° C maximal firmness may not develop within 1 hr. It is of interest that in hemophilia both the clot resistance[5] and the clot firmness are decreased. Blood clots from 2 heparinized rabbits exhibited firmness equal to 10 to 40 mm of Hg which was not affected by 2 hr of incubation.

An exploratory study on clot firmness was done on blood clots of a "dicoumarinized" patient with a history of recurrent thrombophlebitis. Following therapy with 3,3′-methylenebis (4-hydroxycoumarin) the coagulation time and prothrombin time were prolonged, whereas the bleeding time and clot resistance were normal. The clot firmness equalled 70 mm of Hg after 40 min incubation and increased to 200 mm of Hg by 80 min. This phenomenon of an increase in clot firmness after 30 min incubation was observed on 3 separate occasions in blood clots from this patient. We believe this could be due to a delayed formation of fibrin, although it is possible that the change might occur subsequently in the fibrin itself. These findings, and those in normal animals, indicate that a clot which forms in a tube continues to increase in firmness for a period of time.

[5] Copley, A. L., and Lalich, J. J., *J. Clin. Invest.*, 1942, **21**, 145.

13912

Influence of Essential Fatty Acid Deficiency on Transport of Fatty Acids into the Liver.*

RICHARD H. BARNES, IRVING I. RUSOFF AND GEORGE O. BURR.

From the Division of Physiological Chemistry, Department of Physiology, University of Minnesota, Minneapolis.

Although it is well recognized that linoleic acid is not synthesized by the animal body and is a dietary essential for several species of laboratory animals there is no evidence as to the metabolic function of this fatty acid. The common observation that linoleic acid is a normal and apparently ever-present constituent of tissue phospholipids led Burr and Burr[1] to consider the possibility that linoleic acid is necessary for the normal metabolic functioning of tissue phospholipids. The nature of the functions of phospholipids is not definitely known, but one well recognized characteristic is the exchange of phosphorus and fatty acids in the phospholipid molecule. Two attempts have been made to study the rate of phospholipid turnover in the tissues of rats suffering from a deficiency of the essential fatty acids, by Hevesy and Smedley-MacLean,[2] using radioactive phosphorus as a tracer, and by Barnes, Miller, and Burr,[3] using labeled (spectroscopically active) fatty acids. In the labeled acid study essential fatty acid deficiency resulted in a slight decrease in the rate of fatty acid incorporation into the phospholipids of the intestinal mucosa, hence it was thought desirable to extend this type of study to other tissues. The present investigation was undertaken to study fatty acid incorporation into the liver phospholipids of rats subjected to a fat deficient regimen.

Details of the various methods employed

* Assistance in the preparation of these materials was furnished by the personnel of the Works Projects Administration, Official Project No. 265-1-71-236, Subproject 612.

[1] Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, 1930, **86**, 587.

[2] Hevesy, G. C., and Smedley-MacLean, I., *Biochem. J.*, 1940, **34**, 903.

[3] Barnes, R. H., Miller, E. S., and Burr, G. O., *J. Biol. Chem.*, 1940, **140**, 773.

TABLE I.
Rate of Incorporation of Labeled Fatty Acids into the Acetone Insoluble (Phospholipid) Lipid Fraction of the Livers of Essential Fatty Acid "Deficient" and "Cured" Rats.

Treatment	Absorption time hrs	No. of rats	Body wt. g	Wt. of liver g	Phospholipid in liver %	$E^{1\%}_{1cm}$ of phospholipids	Labeled fatty acids in phospholipids %
Cured of F.D.	0	7	160	4.45	3.64	10.3	
F.D.	0	6	126	3.95	3.12	7.9	
Cured of F.D.*	8	3	158	4.95	3.17	8.0	
F.D.*	8	3	133	4.30	3.59	8.0	
Cured of F.D.	2	7	162	4.65	3.45	15.7	1.5
F.D.	2	7	129	4.00	3.54	12.3	1.2
Cured of F.D.	8	7	160	4.84	2.72	23.2	3.6
F.D.	8	7	131	4.28	3.19	21.0	3.6
		Controls from stock diet.†					
Normal	0	4	212	4.29	2.82	28.2	
	8	4	207	5.02	2.87	42.3	3.9

*Rats in these two groups received the normal, unconjugated corn oil (Mazola) having an $E^{1\%}_{1cm}$ at 2350 A of approximately 3.0.

†Data from reference 5, p. 250, Table I, Exp. 3.

have been described elsewhere.[4, 5] The conjugated fatty acids of corn oil, prepared as the methyl esters, were employed as the labeled fatty acids. These esters had an extinction coefficient, $E^{1\%}_{1cm}$ at 2350 A, that was 50 times greater than that of the normal liver acetone insoluble lipid fraction. Rats that had been fasted for 24 hours were fed by stomach tube 0.5 cc of the fatty acid esters per square decimeter body surface.[6] After intervals of 2 and 8 hr the rats were killed by etherization. The livers were weighed, ground in a mortar with sand and finally covered with an alcohol-ether mixture (3:1). Lipid extraction was carried out for several hours at boiling temperature under a stream of nitrogen, the alcohol-ether extract was evaporated almost to dryness and residue taken up in petroleum ether. This extract was washed several times with water, and after a partial evaporation the phospholipids were precipitated with acetone. The acetone insoluble lipids were then taken up in moist ether and analyzed spectroscopically for their content of labeled fatty acid. Details of this analytical procedure have been described.[4]

Two separate experiments were carried out in which female weanling rats were placed on a diet composed of casein 12%, sucrose 84.1%, and salts 3.9%,[7] supplemented by daily doses of 0.7 g of dried yeast and concentrates of vitamins A, D, and E.[8] At the end of 3 months when the growth curves had almost reached a plateau, one half of the rats were given a daily supplement of 3 to 4 drops of corn oil (Mazola) for one month in order to bring about a cure of the deficiency symptoms. The remaining rats continued on the fat deficient regimen during this time. Although definitely improved as to growth and skin conditions none of the rats was com-

[4] Miller, E. S., Barnes, R. H., Kass, J. P., and Burr, G. O., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 485.

[5] Barnes, R. H., Miller, E. S., and Burr, G. O., *J. Biol. Chem.*, 1941, **140**, 247.

[6] Carman, G. G., and Mitchell, H. H., *Am. J. Physiol.*, 1926, **76**, 380.

[7] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, **33**, 63.

[8] Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, 1929, **82**, 345.

pletely cured by this treatment, and for comparative purposes a control group of female rats which had been raised on a stock diet was included in the summary of data presented in Table I. The data for this added control group were taken from a previous publication.[5]

No significant difference exists between the fat deficient and the "cured" animals in the rate of entrance of labeled acids into the acetone insoluble fraction of the liver lipids. Furthermore, the small difference between the two experimental groups and the controls on stock diet is probably well within experimental error.

It is still possible that fatty acid turnover in some tissue phospholipids is affected by essential fatty acid deficiency. Hevesy and Smedley-MacLean[2] found that rats receiving a fat deficient diet showed the same relative turnover of radioactive phosphorus in the kidney and liver phospholipids but greater in muscle phospholipids than did rats which had been cured of the deficiency with linoleic or arachidonic acids. Barnes, Miller, and Burr[3] found a decreased exchange of labeled fatty acids in the intestinal mucosa phospholipids of fat-deficient rats. Until more is known about the metabolic role of this fatty acid exchange phenomenon in phospholipids, the significance of small deviations from normal must be a matter of conjecture.

Summary. Rats suffering from a severe deficiency of the essential fatty acids show the same rate of incorporation of labeled fatty acids into the liver phospholipids as do rats which have been cured of this deficiency by the administration of 3 to 4 drops of corn oil for 1 month.

The rate of incorporation of labeled acid into the liver phospholipids is the same for rats raised on the fat deficient diet as has been previously observed for rats receiving a regular mixed stock diet.

13913 P

Production of the Shwartzman Phenomenon with a Sulfonamide Conjugate of a Bacterial Filtrate.*

ISADORE E. GERBER AND MILTON GROSS (Introduced by G. Shwartzman.)

From the Laboratories of the Mount Sinai Hospital, New York City, and the Hudson County Tuberculosis Hospital, Jersey City.

A bacterial filtrate* of meningococcus, previously shown to be capable of producing the Shwartzman phenomenon,[1] was coupled with paraaminobenzenesulfonylacetylimide† by means of a modification of the original Landsteiner method.[2] The resulting conjugate was a highly colored substance, russet and soluble in alkalis, yellow and insoluble in acids. The conjugate was purified by 10 reprecipitations from sodium carbonate solution using dilute acetic acid as the precipitating agent. The final precipitate was suspended in normal saline and dissolved by neutralization with normal sodium carbonate.

Eight experiments were performed on rabbits. In 4 instances the skin was prepared by an intradermal injection of 0.5 cc of the original bacterial filtrate and in the other 4 by the conjugate. A single intravenous injection of 1.0 cc of either the filtrate or the conjugate was given 24 hours later in each instance. Two hours later a hemorrhagic reaction was observed at the site of skin

* Courtesy of Dr. Gregory Shwartzman, Department of Bacteriology, Mount Sinai Hospital, New York City.

† We are indebted to the Medical Research Division, Schering Corporation, Bloomfield, for this compound.

1 Shwartzman, G., *The Phenomenon of Local Tissue Reactivity*, Paul B. Hoeber, Inc., Med. Book Dept., Harper and Brothers, New York, 1937.

2 Landsteiner, L., and Lampl, H., *Z. Immunitaet. Forsch.*, 1917, **26**, 293.

preparation, reaching a maximum intensity in 4 hr. Either the bacterial filtrate or its conjugate could be used for skin preparation or intravenous injection, interchangeably, for production of the phenomenon. Apparently chemical treatment of the bacterial filtrate in the synthesis of the conjugate did not affect its ability to produce the Shwartzman phenomenon.

The property of the bacterial filtrates to combine with chemicals is comparable with that of animal proteins, horse serum, egg albumen, human serum. Further studies are indicated to isolate the specific components in the filtrate responsible for this combination. These might serve as a further link for the understanding of the factors responsible for human and animal sensitization to protein and bacterial substances.

13914 P

Sodium Sulfathiazole Resistant *Shigella paradysenteriae* Flexner and Sonne.

MERLIN L. COOPER AND HELEN M. KELLER.

From the Children's Hospital Research Foundation and the Pediatric Department of the College of Medicine, University of Cincinnati.

Numerous reports have appeared regarding the development of sulfonamide resistance by pneumococci *in vitro* and *in vivo*, and by gonococci. We have not seen a similar report regarding Shigella paradysenteriae.

In the course of our *in vitro* studies we have developed 2 sodium sulfathiazole resistant strains of Shigella paradysenteriae, one each of the Flexner and Sonne types. Sodium sulfathiazole was bactericidal for the parent Flexner strain in a concentration of 80 mg % and for the sulfonamide resistant sub-strain in a concentration of 600 mg %. Sodium sulfathiazole was bactericidal for the parent Sonne strain in a concentration of 300 mg % and for the sulfonamide resistant sub-strain in a concentration of 1000 mg %.

The sulfonamide resistant Flexner strain retained its virulence for white mice (1 M.F.D. being 1.0 cc of 10^{-5} in 3% mucin), but was non-resistant to sodium sulfathiazole *in vivo*. The resistant Sonne strain became non-virulent.

Further studies are planned with these parent and sulfonamide resistant strains and other sulfonamide compounds.

13915

Comparative Pressor Effects of Parahydroxyphenylisopropylamine (Paredrine) and Parahydroxyphenylisopropylmethylamine (Paredrinol).

M. H. Nathanson and Hyman Engelberg.

From the University of Southern California School of Medicine and the Medical Service, Cedars of Lebanon Hospital.

The introduction of new sympathomimetic amines is of interest to the pharmacologist and the clinician. Two recently developed substances of this group which have been described under the names 'paredrine' and 'paredrinol' are of especial interest in that as compared with epinephrine, they are stable, having an action which is prolonged and effective on oral administration. Parahydroxyphenylisopropylamine (Paredrine) has been investigated in this country but the reports have been few in number. Parahydroxyphenylisopropylmethylamine (Paredrinol) which is the N methyl derivative of paredrine (Fig. 1) has received a great deal of attention in the German literature under the trade name "Veritol."[1] Special advantages over other sympathomimetic substances have been ascribed to this compound, and the drug has been recommended as a superior agent in the treatment of shock. In this country, Stead and Kunkel[2] studied extensively the mechanism of the pressor action of paredrinol and concluded that this resulted from one or both of the following mechanisms: (1) a direct vaso-constrictor effect on minute blood vessels, and (2) a primary increase in venous tone causing an increased venous return to the heart and a secondary rise in arterial pressure. Kunkel, Stead and Weiss[3] concluded that paredrinol is a useful drug in the treatment of collapse caused by the pooling of blood in a dilated venous system.

Although there are studies on the pressor effect of paredrine, there are no comparative studies of the two related compounds. Alles and Prinzmetal[4] have shown that paredrine has a more intense pressor action than phenylisopropylamine. Abbott and Henry[5] concluded that paredrine is about twice as effective as ephedrine in raising blood pressure. Altschule and Iglauer[6] found that paredrine was a more effective pressor substance than benzedrine and concluded that the drug may be useful in the treatment of certain types of

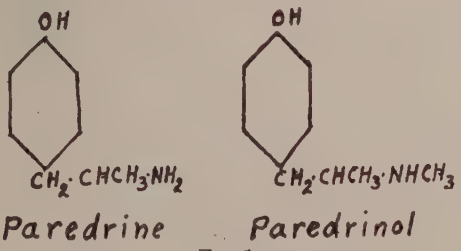


Fig. 1.
Chemical Structure of Paredrine and Paredrinol.

[1] Rein, H., *Arch. f. exp. Path. u. Pharmakol.*, 1937, **187**, 429; Rein, H., *Klin. Wchnschr.*, 1937, **16**, 700; Rein, H., *Arch. f. exp. Path. u. Pharmakol.*, 1937, **187**, 617; Lindner, W., *Arch. f. exp. Path. u. Pharmakol.*, 1937, **187**, 444; Schneider, D., *Klin. Wchnschr.*, 1937, **16**, 736; Robbers, H., *Munchen. med. Wchnschr.*, 1937, **84**, 819; Grosse-Brockhoff, F., and Kaldenberg, F., *Klin. Wchnschr.*, 1937, **16**, 948; Schneider, H., and Kipp, H., *Klin. Wchnschr.*, 1937, **16**, 1672; Schondorf, T., *Munchen. med. Wchnschr.*, 1938, **85**, 333; Klostermeyer, W., and Jonsson, B., *Klin. Wchnschr.*, 1937, **16**, 1724; Meyer, F., and Spiegelhoff, W., *Klin. Wchnschr.*, 1937, **16**, 1342; Grosse-Brockhoff, F., and Kaldenberg, F., *Arch. f. exp. Path. u. Pharmakol.*, 1938, **188**, 383.

[2] Stead, E. A., Jr., and Kunkel, P., *J. Clin. Invest.*, 1939, **18**, 439.

[3] Kunkel, P., Stead, E. A., Jr., and Weiss, S., *J. Clin. Invest.*, 1939, **18**, 679.

[4] Alles, G. A., and Prinzmetal, M., *J. Pharm. and Exp. Therap.*, 1933, **48**, 161.

[5] Abbott, W. O., and Henry, C. M., *Am. J. Med. Sc.*, 1937, **193**, 661.

[6] Altschule, M. D., and Iglauer, A., *J. Clin. Invest.*, 1939, **18**, 476.

TABLE I.
Comparison of the Maximum Pressor Responses (in mm of Hg) Following the Subcutaneous Administration of Paredrine and Paredrinol in Doses of 20 mg and Doses of 40 and 80 mg by Mouth.

	Subcutaneous 20 mg		Oral 40 mg		Oral 80 mg	
	Systolic	Diastolic	Systolic	Diastolic	Systolic	Diastolic
Paredrine	37	14	54	14	82	33
Paredrinol	8	0	40	14	28	18
Paredrine	34	10	60	16	48	10
Paredrinol	8	0	32	6	40	12
Paredrine	58	32	14	6	28	18
Paredrinol	32	4	6	0	11	2
Paredrine	30	10	25	16	78	28
Paredrinol	5	2	16	17	30	18

shock. Nathanson[7] demonstrated that paredrine was very effective in the prevention of the cardiac standstill which may be induced in man by pressure on the carotid sinus. In several instances, studying the comparative effects of paredrine and paredrinol by this method it was found that paredrine was definitely more active. It seemed desirable, therefore, to study the comparative pressor actions of these compounds.

Procedure. Fifteen subjects were studied from the hospital wards. These consisted chiefly of convalescent post-operative patients who were confined to bed. The paredrine was administered as the hydrobromide and the paredrinol as the sulphate.* The studies were made under basal conditions, and were all carried out in the morning with breakfast omitted. Repeated observations were made under the same conditions. After several control readings of the blood pressure the drug was administered, and subsequent blood pressure readings were made at 5, 15, 30, 45 and 60 min after the subcutaneous administration; and 15, 30, 60 and 90 min; and in 2 instances, 2 hr after the oral administration. After a subject had received one of the drugs, at least 24 hr elapsed before studies were repeated with the second compound. Fifteen subjects received both drugs subcutaneously in doses of 20 mg. Nine subjects had both compounds by mouth in doses of 40 mg and 4 of these also received 80 mg by mouth.

Table I shows the results in 4 subjects receiving 20 mg subcutaneously and 40 and 80 mg by mouth.

Figure 2 shows the blood pressure responses in 4 subjects after the subcutaneous administration of paredrine and paredrinol.

Results. In each instance a rise in arterial pressure was observed which varied greatly in different individuals. In every case, paredrine showed a much greater rise of pressure than paredrinol. On oral administration, the pressor effect was observed in 15 minutes in most instances, and the maximum effect occurred in 30 to 60 min. The duration of the pressor action with the 40 mg dose of paredrine was 60 to 90 min, and with 80 mg one and a half to 2 hr. In each instance Paredrine showed a considerably more prolonged effect than paredrinol. Following the subcutaneous injection, the onset of the effect was within 5 to 10 minutes; and the maximum effect was observed usually within 15 to 30 min. The blood pressure returned to normal in an hour in 13 of the 15 cases. The duration of the effect was again consistently shorter with the paredrinol.

Conclusions. Paredrine and paredrinol have a definite pressor effect in man when administered orally or subcutaneously. The systolic pressure is much more influenced than the diastolic pressure. Paredrine consistently

[7] Nathanson, M. H., *Ann. Int. Med.*, 1939, **12**, 1855.

* The Paredrine Hydrobromide was supplied by the Smith, Kline and French Laboratories of Philadelphia, and the Paredrinol Sulphate by Dr. Gordon Alles of Pasadena.

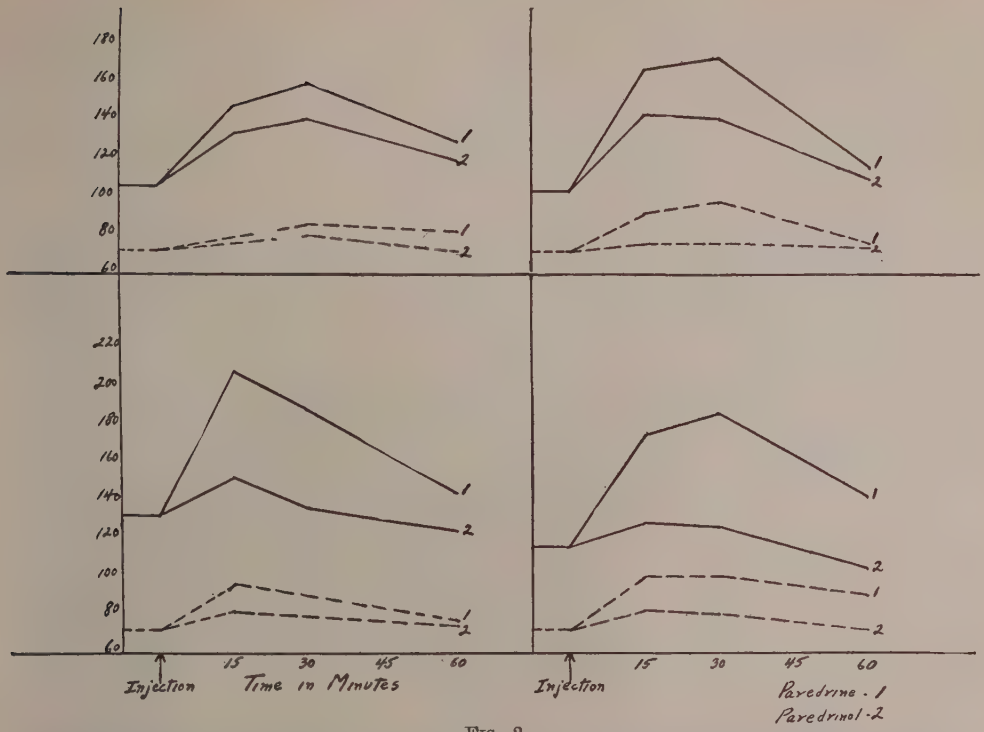


FIG. 2.

Typical course of blood pressure reaction following the subcutaneous administration of 20 mg of paredrine and 20 mg of paredrinol in 4 subjects. (1) Represents the response to paredrine and (2) the response to paredrinol. Solid lines indicate the systolic pressures and broken lines the diastolic pressures.

shows a much greater and more prolonged pressor action than paredrinol. There is a marked variation in response in different individuals to a given dose.

A comparison of the results of oral and subcutaneous administration in the same individual indicates in most instances a complete or practically complete absorption and utilization of these compounds when administered by mouth.

13916

Effect of Paredrine on Blood Specific Gravity and Blood Volume.

HYMAN ENGELBERG AND M. H. NATHANSON.

From the University of Southern California School of Medicine and the Medical Service, Cedars of Lebanon Hospital.

From the preceding report it was demonstrated that paredrine (parahydroxyphenylisopropylamine) has a more intense pressor action in human subjects than paredrinol (parahydroxyphenylisopropylmethylamine), a sympathomimetic compound which has been recommended in the treatment of shock.[1]

In most forms of shock there is a definite hemoconcentration and a reduction in blood volume. The hemoconcentration is demonstrable by a rise in the specific gravity of the blood.[2] Prolonged stimulation of the sympathetic nervous system has been reported to result in a diminution in blood volume and in a condition resembling shock.[3] It seemed important, therefore, before applying a sympathomimetic drug in the treatment of shock to determine the influence of the drug on blood concentration and blood volume.

I. *Specific gravity of the whole blood.* In the present studies, the specific gravity of the blood was determined by the falling drop method of Barbour and Hamilton.[4] The specific gravity was measured before the administration of the drug and at the height of the pressor effect following the subcutaneous administration of 20 mg of paredrine hydrobromide in 5 subjects. Table I summarizes the results of these experiments. In 4 instances there was a slight rise in the specific gravity of the blood after the administration of the drug. The variations noted do not, however, indicate any significant degree of hemoconcentration.

II. *The effect on blood volume.* The "Evans Blue" dye was employed in determining the blood volume following the method described by Gibson and Evans.[5] The dye concentration in the serum was estimated with a photo-electric cell colorimeter. Paredrine hydrobromide was administered in three subjects in large doses so that a marked and prolonged pressor effect was obtained. The drug was administered subcutaneously in doses of 30 mg in 2 subjects; in 40 mg doses in one instance. In each case 3 control readings of the dye concentration of the plasma was made during the hour preceding the administration of the drug, to estimate the normal curve of disappearance of dye from the blood. Readings were again made 10 min after the administration of the paredrine, at the height of the pressor effect, usually between 20 and 25 minutes, and at the completion of the pressor effect which varied from 90 to 165 min after the drug was administered.

TABLE I.

Blood Pressure Readings and Specific Gravity Determinations Following the Subcutaneous Administration of 20 mg of Paredrine Hydrobromide. The readings were made at 15, 30, and 60-minute periods.

	Control	15′	30′	60′
Blood pressure	148/94	200/96	212/98	168/96
Specific gravity	1.0564		1.0569	
B.P.	128/40	160/56	188/70	148/40
Sp.Gr.	1.0492		1.0493	
B.P.	105/70	166/92	170/98	114/75
Sp.Gr.	1.0526		1.0532	
B.P.	132/72	205/96	188/92	146/76
Sp.Gr.	1.0581		1.0581	
B.P.	115/85	172/100	182/100	144/90
Sp.Gr.	1.0513		1.0520	

Table II shows the blood pressure and blood volume before and after the administration of the paredrine. There was no sig-

[1] Rein, H., *Arch. Exp. Path. u. Pharmacol.*, 1937, **187**, 617.

[2] Moon, H., *Ann. Int. Med.*, 1939, **13**, 451.

[3] Freeman, N. E., *Am. J. Physiol.*, 1933, **103**, 185.

[4] Barbour, H. G., and Hamilton, W. F., *J. A. M. A.*, 1927, **88**, 91.

[5] Gibson, J. G., 2d, and Evans, W. A., *J. Clin. Invest.*, 1937, **16**, 301.

TABLE II.
Blood Pressure Readings and Blood Volume Determinations Following the Subcutaneous Administration of Paredrine Hydrobromide. Patients B.C. and J.M. received 30 mg and H.C. received 40 mg.

		Before drug at		After the drug at		
		30 min	15 min	15 min	30 min	90 min
B.C.	Blood pressure	118/80	122/80	164/88	172/100	110/74
	Total blood volume, cc	5000	5000	4975	5000	5041
J.M.	Blood pressure	120/70	120/70	168/86	194/112	130/72
	Total blood volume, cc	5855	5840	5770	5855	5870
H.C.	Blood pressure	118/72	120/70	210/110	208/110	150/80
	Total blood volume, cc	5206	5200	Specimen clotted	5265	5200

Paredrine subcutaneously.

nificant deviation in the blood volume. There was also no significant change in the hematocrit readings following the administration of the drug.

Conclusion. The administration of paredrine in doses sufficient to produce a definite and prolonged rise in blood pressure has no influence on the specific gravity of the blood and blood volume in normal individuals.

13917

Relative Ineffectiveness of Arsenocholine as a Methylating Agent in the Chick.

H. J. ALMQUIST AND T. H. JUKES.*

From the Division of Poultry Husbandry, University of California, Berkeley and Davis.

Arsenocholine is an effective substitute for choline as a growth-promoting and antiperotic factor for the turkey and the chick.[1] In the rat, arsenocholine may assume several of the functions of choline, such as a lipotropic activity, but fails to exhibit appreciable methylating ability. The literature on the metabolism of arsenocholine in the rat has been concisely reviewed.[2] In seeking further explanation of the mechanism of growth-promoting and antiperotic effects of arsenocholine in the fowl it seemed of interest to determine whether this analogue of choline could exhibit a methylating action in such species. Since it was known that choline is capable of methylating homocysteine in the chick,[3] studies were made of the comparative activity of arsenocholine in this respect.

White Leghorn chicks were placed at hatching time on a low-choline diet[4] and maintained on this diet for 14 days in order to deplete them of choline. At 10 days of age they were carefully selected for uniform weight and vigor and divided into groups of 4 each. The individual growth rates were measured for 4 more days. All groups maintained uniform and comparable rates of growth during the latter period. They were then given the experimental diets for 8 days. A few chicks not selected for the experimental groups were kept on the low-choline diet.

* Present address Lederle Laboratories, Inc., Pearl River, N.Y.

1 Jukes, T. H., *J. Nutrition*, 1940, **20**, 445; Jukes, T. H., and Welch, A. D., *J. Biol. Chem.*, in press.

2 Welch, A. D., and Landau, R. L., *J. Biol. Chem.*, 1942, **144**, 581.

3 Klose, A. A., and Almquist, H. J., *J. Biol. Chem.*, 1941, **138**, 467.

4 Jukes, T. H., *J. Nutrition*, 1941, **22**, 315.

TABLE I.

Effects of Choline and Arsenocholine on the Utilization of Homocystine for Growth of the Chick.

Pen	Diet	Supplement	Level, %	Avg % gain per day*
1	Basal arachin diet	Choline chloride	.50	1.7
2	" " "	dl-homocystine	.75	3.2
3	" " "	dl-homocystine Arsenocholine chloride	.75 .50	3.5
4	" " "	dl-homocystine Choline chloride	.75 .05	3.3
5	" " "	dl-homocystine Arsenocholine chloride Choline chloride	.75 .50 .50	5.6
6	" " "	dl-methionine Choline chloride	.60 .50	6.8
7	Low-choline diet	Choline chloride l-cystine	.20 .25	6.5
8	Practical rearing diet	None	—	6.9

*Gain in weight per day divided by mean weight for the test period and multiplied by 100.

These chicks developed a high incidence of perosis due to choline deficiency.

The basal arachin diet contained per 100 parts, calcium gluconate, 8; tricalcium phosphate, 2; dipotassium phosphate, 0.5; potassium chloride, 0.3; sodium chloride, 1; manganese sulphate monohydrate, 0.03; sodium silicate, 0.2; potassium iodide, 0.01; ferric oxide, 0.02; copper sulphate, 0.005; zinc sulphate, 0.005; magnesium sulphate, 0.3; Cellu-flour (cellulose), 5; 2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester (sodium salt), 0.001; biotin concentrate (SMACO No. 1000), 0.05; yeast extract (1:4), 1; thiamin, 0.001; riboflavin, 0.001; pyridoxine, 0.001; nicotinic acid, 0.005; calcium pantothenate (dl), 0.006; cod liver oil (U. S. P.), 1; cholic acid, 0.05; deoxycholic acid, 0.1; vitamin E. distillate, 0.025; Wesson oil, 2.5; gelatin, 7; arachin, 23; d-lysine, 0.1; dl-threonine, 0.2; l-tryptophane, 0.3; l-cystine, 0.2; dl-valine, 0.5; l-tyrosine, 0.2; and Cerelose (glucose) to complete 100 parts.

The sample of gelatin used contained less than 0.1% methionine. The arachin may have contained up to 0.67% methionine and 1.51% cystine.[5] The basal arachin diet could not have contained appreciably more than 0.16% methionine and 0.55% cystine. Supplements to this diet and the results obtained are given in the table. The extent of these studies was necessarily limited by the amounts of arsenocholine and homocystine available.

Chicks fed the arachin diet supplemented with methionine and choline (pen 6) were able to grow at a rate equivalent to that of chicks fed the low-choline diet plus choline (pen 7) or the practical rearing diet (pen 8). This rate of gain, approximately 7% per day, is normal for the strain of chicks and the age period involved.

Addition of choline to the arachin diet led to only a very low rate of gain (pen 1). Addition of homocystine resulted in a somewhat greater rate of gain (pen 2), although less than one-half the normal, indicating that the chicks were able to utilize the homocystine to some extent in spite of the attempted depletion in choline and the absence of a choline supplement in the diet. The presence of ample quantities of cystine in the basal diet would seem to indicate that the small growth-promoting effect of the homocystine did not involve its conversion to cystine. It is more probable that some of the homocystine was methylated.

An essentially complete utilization of homocystine in the chick requires a choline intake

[5] Brown, W. L., *J. Biol. Chem.*, 1942, **142**, 299.

which is several times the minimum requirement for growth, perhaps as much as 0.5% of the diet.[3] It was felt, therefore, that any methylating power of arsenocholine would not be observed at dietary levels lower than 0.5%. The results obtained with the combination of homocystine and arsenocholine (pen 3) did not indicate that the utilization of homocystine was appreciably increased. A minimum addition of choline (pen 4) also did not result in an appreciable increase in the utilization of homocystine. However, an addition of 0.5% choline chloride to the combination of 0.5% arsenocholine chloride and homocystine did effect a very appreciable increase in rate of gain (pen 5).

This latter result showed that a sufficient level of choline was capable of increasing the rate of gain in the presence of arsenocholine which, in itself, was comparatively ineffective. The rate of gain, 5.5%, was somewhat short of the optimal rate, which may suggest a mildly toxic action of arsenocholine. Thus, in the case of Group 3, the possibility exists that a slight methylating capacity of arsenocholine may have been almost exactly compensated for by a mild toxic action. It was also observed, however, that chicks reared from date of hatching to 14 days on the low-choline diet plus 0.5% of arsenocholine chloride made gains that were approximately normal, hence it does not seem that the arsenocholine could have been seriously detrimental to growth. Chicks fed arsenocholine possessed a strong odor of garlic, probably due to the exhalation of trimethylarsine[2, 3]

Summary. Arsenocholine as compared to choline is relatively ineffective as a methylating agent for homocysteine in the chick.

The authors are indebted to Dr. A. D. Welch for the arsenocholine, to Merck and Company, Inc., for some of the amino acids added to the diets, to Distillation Products, Inc., for the vitamin E concentrate, and to Riedel-deHaen, Inc., for the bile acids.

13918

In Vitro Effect of Urea-Sulfathiazole Combination on Sulfathiazole-resistant Staphylococci.*

HENRY M. TSUCHIYA, DANIEL J. TENENBERG,† ERNEST A. STRAKOSCH AND WILLIAM G. CLARK.

From the Department of Bacteriology, Division of Dermatology and Syphilology, and Department of Zoology, University of Minnesota.

Several investigators have reported that under certain conditions, *Diplococcus pneumoniae, Brucella, Streptococcus, Staphylococcus, Neisseria gonorrheae, Escherichia coli,*[1-6] and possibly other organisms, develop resistance to sulfonamides ("sulfonamide-fastness"). It has been suggested that the sulfonamide-fastness is due to the production

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We wish to thank Doctor Weslie W. Spink and Mrs. Jean J. Vivino for furnishing us with the basal synthetic medium and Strain 14C used in this investigation.

† Research Fellow on above grants.

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3 Neter, E., *Am. J. Surg.*, 1942, **58**, 69.

4 Vivino, J. J., and Spink, W. W., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 336.

5 Stokinger, H. E., Charles, R. C., and Carpenter, C. M., *J. Bact.*, 1942, **44**, 216.

6 Schmelkes, F. C., and Wyss, O., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 263.

TABLE I.

In Vitro Effect of Urea and Sulfathiazole on Sulfathiazole-fast Staphylococci.

Strain of Staph.	Organisms per ml × 1000					
	14c		S2S1		S9S2	
Experiment	1	2	1	2	1	2
Initial population	.7	.7	1.5	1.5	2.9	2.9
Plain control	500,000	500,000	128,000	197,000	450,000	630,000
SAT* control	500,000	550,000	169,000	184,000	590,000	600,000
Urea controls						
Urea 1.25%	1,380,000	1,360,000			350,000	680,000
Urea 1.75%			137,000	152,000		
SAT + urea						
SAT; urea 1.25%	13,400	1,400				
SAT; urea 1.75%			3,300	5,000	53,000	44,000

*SAT—Sodium sulfathiazole; 60 mg%.

of sulfonamide inhibitors by the organisms.[7]

In previous work[8] and in another place in this issue, we have shown that a combination of urea and sulfonamide exerts, in the presence of sulfonamide inhibitor, a greater bacteriostatic activity against *E. coli* than does sulfonamide alone in the presence of inhibitors.

These facts prompted us to investigate the effect of sulfonamide-urea combinations on sulfonamide-fast *Staphylococcus*.

Azochloramid-sulfonamide combinations,[9,3,6] and adenine or hypoxanthine-sulfonamide combinations *in vitro*[10] have been shown to exert greater bacteriostatic effect in the presence of sulfonamide-inhibitors than sulfonamides alone in the presence of inhibitors.

Experimental. Three sulfonamide-fast strains of *Staphylococcus aureus* were used. Stain 14 C was kindly given to us by Dr. W. W. Spink. This strain had been made sulfonamide-fast in his laboratory.[4] Strains S2S1 and S9S2 were isolated by one of us‡ from human abscesses infected with staphylococci, which had failed to respond to prolonged sulfonamide treatment. Strakosch and Clark[11] have discussed these clinical cases in which urea-sulfonamide mixtures had been used. The basal synthetic medium used is described by Gladstone.[12]

Preliminary experiments were carried out to establish the concentrations of urea and sodium sulfathiazole which were without inhibitory effect on growth.

At the time of inoculation the pH values of the test cultures were 7.2-7.3 as determined by the glass electrode. Terminal pH values were also determined.

Ten ml of medium containing the test substances were inoculated with 24-hour cultures of the strains of staphylococci described. After incubation at 37° C for 24 hr, these cultures were plated out in nutrient agar, 5 replicate plates per test culture. The plates were counted after incubation at 37° C for 48 hr.

Results. The means of the 5 replicate plates for the various test cultures are given in Table I.

Table I shows that under the conditions of the experiments, (1) the concentrations of urea used had no significant inhibitory effect on the growth of the organisms, (2) the concentration of sulfathiazole used (60 mg %) had no significant effect on the growth of the organisms, (3) urea-sulfathiazole combination caused a marked inhibition of growth in each case.

7 Mirick, G. S., *J. Clin. Invest.*, 1942, **21**, 628P.

8 Tsuchiya, H. M., Tenenberg, D., Clark, W. G., and Strakosch, E. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 262.

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10 Harris, J. S., and Kohn, H. I., *J. Biol. Chem.*, 1941, **141**, 989.

‡ Ernest A. Strakosch, M.D.

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12 Gladstone, G. P., *Brit. J. Exp. Path.*, 1939, **20**, 189.

Discussion. The strains of staphylococci used in these experiments are resistant to the bacteriostatic action of sodium sulfathiazole. It might be noted that strain 14 C was made sulfonamide-fast *in vitro,* while the other 2 strains were sulfonamide-fast when isolated from sulfonamide-treated patients.

The parent strain of 14 C and 2 additional strains from our laboratory museum all proved to be susceptible to 5 mg % of sodium sulfathiazole, under conditions identical to those used in these experiments.

The terminal hydrogen ion concentrations (pH 7.3 to 6.9) varied in direct proportion to the amount of growth and since the initial pH values of the various test cultures were identical (pH 7.2-7.3), it is indicated that the results observed may not be due to the effect of hydrogen ion concentration upon sulfonamide ionization. It has been claimed by several workers[13–15] that the bacteriostatic efficiency of sulfonamides is influenced by the degree of their ionization.

The mechanism of sulfonamide-fastness is not completely understood. Some workers have claimed that sulfonamide-fastness is due to the production of sulfonamide-inhibitors such as p-amino benzoic acid by the organisms.[7] Whatever the cause of sulfonamide-fastness may be, the results noted here indicate that in the concentrations used, sodium sulfathiazole-urea combinations exert a bacteriostatic effect whereas sodium sulfathiazole alone does not.

Summary. Three strains of sulfathiazole-resistant staphylococci in a synthetic medium, were found to be susceptible to urea-sodium sulfathiazole combinations whereas they were unaffected by these two agents used separately, under the conditions of the experiments described.

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15 Cowles, P. B., *Yale J. Biol. and Med.*, 1942, **14**, 599.

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In vitro Effect of Sulfonamides plus Urea on *Escherichia coli* in Presence of Para-aminobenzoic acid.*

DANIEL J. TENENBERG,† HENRY M. TSUCHIYA, WILLIAM G. CLARK AND ERNEST A. STRAKOSCH.

From the Departments of Bacteriology and Zoology, and Division of Dermatology and Syphilology, University of Minnesota.

At the time that studies on sulfonamides were being initiated, it was noticed by the earlier workers that certain substances inhibited the bacteriostatic action of these drugs. Methionine and p-aminobenzoic acid are among these inhibitors.[1] In a previous paper[2] it was shown that in the presence of methionine, urea and sodium sulfadiazine will inhibit the growth of *Escherichia coli,* whereas in the presence of inhibitor, sodium sulfadiazine alone had little or no bacteriostatic action. To see if urea and sodium sulfadiazine, and urea and other sulfonamides, would

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† Research Fellow on above grants.

1 Dubos, R. J., *Ann. Rev. Biochem.*, 1942, **11**, 659.

2 Tsuchiya, H. M., Tenenberg, D. J., Clark, W. G., and Strakosch, E. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 262.

exert a bacteriostatic effect on *E. coli* in the presence of another sulfonamide inhibitor, p-aminobenzoic acid, the present experiments were carried out.

Experimental. A freshly isolated strain of *E. coli* was used in these experiments. Before the tests were run, the organism was first serially transferred several times in the synthetic medium, the composition of which is given in our previous paper.[2]

Preliminary experiments were performed to establish the tolerance range of our organism in the synthetic medium, to sodium sulfadiazine, sodium sulfathiazole, sulfanilamide, urea, and p-aminobenzoic acid. Various ratios of concentrations of the inhibitor and the sulfonamides were tested to determine the least amount of p-aminobenzoic acid which would be necessary to exhibit anti-sulfonamide activity.

The initial pH of the synthetic medium plus the various substances was 6.8 ± 0.1, as measured by the glass electrode just prior to inoculation. The terminal pH values were also determined.

Twenty-five ml amounts of the synthetic medium containing the various test substances were inoculated with 0.1 ml of a 24 hr synthetic medium culture. These cultures were incubated at 37° C for 24 hr, and the populations were determined by plating out in nutrient agar medium, using 5 replicate plates per test culture. The plates were counted after incubation at 37° C for 48 hr.

Results. The means of the 5 replicate plates for the various test cultures are given in Table I.

Table I shows that, under our experimental conditions, (1) Urea alone, in the concentrations used, had no significant effect on growth, (2) sulfanilamide, and the sodium salts of sulfathiazole and sulfadiazine alone, in the concentrations used, markedly inhibited growth, (3) p-aminobenzoic acid, in the concentrations used, significantly inhibited sulfonamide bacteriostasis, (4) combinations of urea and sulfonamides were bacteriostatically effective, in the presence of p-aminobenzoic acid.

Discussion. These experiments extend the

TABLE I.

In Vitro Effect of Sulfonamides and Urea on *E. coli* in Presence of Para-aminobenzoic Acid.

Experiments with	Populations expressed in millions per ml					
	SAN		SAT		SAD	
	1	2	1	2	1	2
Control	372	334	211	200	135	140
Urea controls						
Urea 4%			225	241	165	155
" 6%	299	272				
Sulfonamide controls						
SAN 15.0 mg%	67	55				
SAT 1.0 "			12.9	11.9		
SAD 1.0 "					19.4	23.1
Sulfonamide-p-aminobenzoic acid controls						
SAN 15 mg%; PAB .001 mg%	279	248				
SAT 1 " ; PAB .02 "			165	145		
SAD 1 " ; PAB .02 "					55	86
Sulfonamide-p-aminobenzoic acid-urea						
SAN 15 mg%; PAB .001 mg%; urea 6%	38	34				
SAT 1 " ; PAB .02 " " 4%			30.1	33.3		
SAD 1 " ; PAB .02 " " 4%					26	32

SAN—sulfanilamide.
SAT—Na-sulfathiazole.
SAD—Na-sulfadiazine.
PAB—p-aminobenzoic acid.

observations made in our previous work,[2] in which methionine was used as the inhibitor, to p-aminobenzoic acid as the inhibitor. Furthermore, using this inhibitor, 2 additional sulfonamides, sulfanilamide and sodium sulfathiazole, showed the same effect, whereas sodium sulfadiazine was the sulfonamide selected in the previous work.

The hydrogen ion concentration values determined at the time of plating, in general were found to increase with the amount of growth. This indicates that the urea effect may not be due to sulfonamide ionization. This point is being studied further. Fox and Rose,[3] Schmelkes, Wyss, *et al.*,[4] Cowles,[5] and others,[6, 7] claim that the bacteriostatic efficiency of sulfonamides is influenced by their degree of ionization.

The mode of action of urea is not as yet elucidated. It is not known whether or not urea acts similarly to azochloramid and certain purines such as adenine and hypoxanthine, which when used in conjunction with sulfonamides, were bacteriostatically effective in the presence of sulfonamide inhibitors.[8-11]

Summary. The concentrations of sulfanilamide, sodium sulfathiazole and sodium sulfadiazine which were required to exert bacteriostatic effect on *Escherichia coli* in a synthetic medium, were determined. Concentrations of para-aminobenzoic acid which caused a significant anti-sulfonamide activity were determined. Concentrations of urea which had no effect on bacterial growth were also determined.

Combinations of these concentrations of sulfonamides and urea, proved to possess bacteriostatic action even in the presence of the sulfonamide inhibitor.

3 Fox, C. L., Jr., and Rose, H. M., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 142.

4 Schmelkes, F. C., Wyss, O., Marks, H. C., Ludwig, B. J., and Strandskov, F. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 145.

5 Cowles, P. B., *Yale J. Biol. and Med.*, 1942, **14**, 599.

6 Tolstoouhov, A. V., *Proc. Am. Chem. Soc.*, Sept., 1942.

7 Roblin, R. O., Jr., and Bell, P. H., *Proc. Am. Chem. Soc.*, Sept., 1942.

8 Neter, E., *J. Bact.*, 1942, **44**, 261.

9 Neter, E., *Am. J. Surg.*, 1942, **58**, 69.

10 Schmelkes, F. C., and Wyss, O., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 263.

11 Harris, J. S., and Kohn, H. I., *J. Biol. Chem.*, 1941, **141**, 989.

13920

On the Adenosine Triphosphate-Myosin System.

MORRIS ZIFF. (Introduced by E. Chargaff.)

From the Department of Biochemistry, College of Physicians and Surgeons, New York City.

Recent data indicate that the chemical energy resulting from the breakdown of carbohydrate in muscle is converted to the mechanical energy required for muscle contraction by means of a reaction between adenosine triphosphate and myosin.[1] Engelhardt and Ljubimova have shown that this is an enzymatic reaction during which adenosine triphosphate is hydrolyzed to adenosine diphosphate and inorganic phosphate.[2] Needham *et al.* suggest that this reaction is associated with the stretching of the myosin fiber from the contracted state, to which it returns on nerve stimulation.[3] Since the adenosine triphosphate-myosin system is so intimately related to the contraction of the myosin fibrils,

1 Kalckar, H. M., *Chem. Rev.*, 1941, **28**, 71.

2 Engelhardt, W. A., and Ljubimova, M. N., *Nature*, 1939, **144**, 668.

3 Needham, J., *et al.*, *Nature*, 1941, **147**, 766.

TABLE I.
Hydrolysis of Adenosine Triphosphate by Myosin in Presence of Added Substances.

No.	System*	Mg inhibitor added	Total P liberated in γ	γ P liberated enzymatically	% enzymatic hydrolysis
1	A.T.P.	—	9	—	0
2	A.T.P. + Myosin	—	17	8	14
3	A.T.P. + Ca + Myosin	—	27	18	31
4	A.T.P. + Ca + Myosin + Acetylcholine Bromide	0.1	27	18	31
5	A.T.P. + Ca + Myosin + Ethoxycholine Bromide	0.1	27	18	31
6	A.T.P. + Ca + Myosin + Curare†	0.1	28	19	33
7	A.T.P. + Ca + Myosin + Zinc Chloride	1.0	9	0	0
8	A.T.P. + Ca + Myosin + Copper Sulfate	1.0	10	1	1.7

*1.2 mg of the barium adenosine triphosphate were added in each experiment as the sodium salt. The time of reaction was 12 minutes.

† The curare, Squibb Intocostrin, was obtained in solution from E. R. Squibb and Sons, New York City. Its concentration is based upon physiological assay.

it was considered of interest to study the effects of chemical mediators of nerve stimulation, acetylcholine and epinephrine, which influence muscle contraction, on the velocity of this reaction with a view toward elucidating the site of action of these substances.

It was observed that the liberation of inorganic phosphate was entirely uninfluenced by any of the following substances: acetylcholine, ethoxycholine, epinephrine, eserine, atropine, curare, nicotine, cysteine, heparin, fluoride, thiocyanate, and cyanide. Only when copper* and zinc salts were added was the reaction inhibited. It would appear, therefore, that the point of action of such muscle stimulators as acetylcholine, epinephrine, curare, etc. is not the adenosine-triphosphate system, at least as it was studied *in vitro*.

Myosin was prepared by the method of Greenstein and Edsall.[5] † The monobarium salt of adenosine triphosphate was prepared according to Kerr.[7] It had a total phosphorus content of 13.1%, an easily hydrolyzable phosphorus content of 8.6%, and was free of inorganic phosphate. Before each experiment it was converted to the sodium salt by treatment with sodium sulfate. The runs were carried out in borate buffer of pH 8.6. To 0.2 cc of a myosin solution containing 2.8 mg N per cc, 1.8 cc of borate buffer, 0.1 cc of a solution of the substance being tested, 0.05 cc of a 0.1 molar calcium chloride solution, and 0.01 cc of a solution containing the equivalent of 1 mg of the barium salt of adenosine triphosphate were added. This system was incubated at 37°. After a specific interval which varied from 12 to 25 minutes in different experiments, 2 cc of a 10% trichloracetic acid solution were added and the protein precipitate was removed by centrifugation. Inorganic phosphate was determined in the supernatant solution by the method of Fiske and Subbarow.[8] In some experiments the calcium chloride was omitted. Appropriate control experiments were carried out without myosin and without adenosine triphosphate.

In Table I are given results obtained in a typical experiment. It is apparent that the organic substances tested were inactive. When studied in the same manner, epinephrine

* The inhibiting effect of copper salts has recently been reported by Bailey.[4]

[4] Bailey, K., *Biochem. J.*, 1942, **36**, 121.

[5] Greenstein, J. P., and Edsall, J. T., *J. Biol. Chem.*, 1940, **133**, 397.

† It was considered desirable for the purpose of the present experiments to avoid more than one precipitation of the myosin. It may be expected, for this reason, that it contained significant amounts of adenosine diphosphatase.[4,6]

[6] Ljubimova, M. N., and Pevsner, D., *Biochimia*, 1941, **6**, 178.

[7] Kerr, S. E., *J. Biol. Chem.*, 1941, **139**, 121.

[8] Fiske, C. H., and Subbarrow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

hydrochloride, eserine sulfate, atropine sulfate, nicotine and sodium fluoride, in 100 γ quantities, and cysteine hydrochloride, potassium thiocyanate, potassium cyanide, and the sodium salt of heparin‡ in 1 mg quantities were also inactive.

Summary. Acetylcholine, epinephrine, a series of alkaloids, and several other substances were without influence on the hydrolysis of adenosine triphosphate. Copper and zinc salts inhibited the reaction.

‡ This was obtained from Hoffmann-La Roche and Co., Nutley, N.J.

13921 P

In vivo Activity of Streptothricin Against *Brucella abortus.**

H. J. Metzger, Selman A. Waksman and Leonora H. Pugh.
From the New Jersey Agricultural Experiment Station, New Brunswick, N.J.

An antibiotic substance possessing rather strong bacteriostatic and bactericidal properties against many gram negative and certain gram positive bacteria has been isolated from a soil *Actinomyces* by Waksman and Woodruff[1] and designated as streptothricin. This substance has now been tested extensively against *Brucella abortus,* the causative agent of brucellosis, and was found to have a strong inhibitory action against this organism not only *in vitro,* but also *in vivo.*

Bactericidal tests of the action of streptothricin† were made by adding graduated doses of the crude preparation of this substance to 10 cc portions of a suspension of *Br. abortus* in nutrient broth and incubating 2 hr at 37°C. This incubated suspension was then plated on tryptose agar. No growth of the organism was obtained on plates inoculated from the preparations containing 0.1 mg or more of the crude streptothricin per 10 cc of broth.

The *in vivo* tests of the activity of streptothricin against *Br. abortus* were at first conducted with incubating eggs. Toxicity tests indicated that the chick embryo tolerated enough of the crude preparation of this substance to warrant testing its activity against this pathogen in such a living medium.

A series of groups of 15-day incubated eggs were inoculated on the chorioallantoic membrane with approximately 2,000 *Br. abortus* cells, using 48-hr-old cultures of Strain No. 19 grown on tryptose agar, suspended in saline solution. Cultures made from control groups of eggs 4 to 5 days after inoculation indicated this to be an infective dose. On the day following the inoculation of the eggs, those scheduled for treatment were given varying amounts of streptothricin, all of which were below the toxic level. The material was administered on the chorioallantoic membrane. These treated eggs were cultured on the 19th or 20th day of total incubation. The results showed that 10 mg of crude streptothricin, administered 24 hr after the inoculation of the eggs with *Br. abortus,* had been sufficient to bring about complete destruction of this organism in the living chick embryo.

These experiments with chick embryos were followed by other experiments employing guinea pigs. At first, an initial dose of 100 mg of crude streptothricin was administered simultaneously with the inoculation of the animal with a small infective dose of *Br. abortus.* The streptothricin was given intraperitoneally to one group and subcutaneously to another group of animals, whereas the controls were left untreated.

* Journal series paper, New Jersey Agricultural Experiment Station, Rutgers University, Departments of Dairy Husbandry and Soil Microbiology.

1 Waksman, S. A., and Woodruff, H. B., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 207.

† We are indebted to Merck and Company, Rahway, N.J., for supplying some of the streptothricin used in this work.

Following the initial dose, this treatment was continued for 4 weeks at 5-day intervals, so that each guinea pig received a total of 600 mg of the crude streptothricin. After 4 weeks all 3 groups of animals were bled from the heart and killed for culturing. The blood serum of the treated groups showed no *Br. abortus* agglutinins in a 1:25 dilution, whereas that of the control group agglutinated completely in dilutions varying from 1:25 to 1:400. *Br. abortus* was recovered from all untreated controls but from none of the treated guinea pigs.

In another experiment, the treatment with streptothricin was started one week after the inoculation of the guinea pigs with approximately 80,000 *Br. abortus* cells. A total of 1200 mg of crude streptothricin was administered to each animal intraperitoneally at regular intervals over a period of 3 weeks, after which the treated and the control animals were examined as in the previous experiment. The blood serum of the treated animals showed no *Br. abortus* agglutinins in a 1:12.5 dilution, whereas that of the controls agglutinated completely in dilutions varying from 1:50 to 1:400. *Br. abortus* was recovered from the spleens of both the treated and the control groups but plate counts made from ground splenic pulp indicated that there was a much greater concentration of organisms in the spleens of the untreated controls than in the treated group.

Summary. Streptothricin, an antibiotic substance obtained from a soil *Actinomyces*, has been tested against *Brucella abortus* both *in vitro* and *in vivo* with favorable results. Experiments with incubating eggs established that the toxicity of streptothricin is low enough to make possible the administration of doses sufficient to destroy *Br. abortus* in the living tissues. Studies with guinea pigs indicated that streptothricin offers considerable promise as an antibiotic agent against brucellosis in animals.

13922 P

A New Method for Separation of the Basic Amino Acids from Protein Hydrolysates.*

RICHARD J. BLOCK.
Cooper Road, Scarsdale, N.Y.

Four methods have been described for the separation of the basic amino acids from the other products of protein hydrolysis. The oldest is by precipitation with phospho-24-tungstic acid according to Hausmann[1] and to Van Slyke.[2] A second is by electrolytical transport introduced by Ikeda and Suzuki[3] and developed by Foster and Schmidt,[4] Kuhn and Desnuelle,[5] and others.[6-8]

Whitehorn[9] showed that arginine, histidine, and lysine were exchanged for Na^+ ions on the synthetic zeolite, "Permutit." An application of this finding is Dubnoff's[10] use of "Permutit" to separate arginine from

* Encouragement in carrying out this project was received from the Food Branch, War Production Board.

1 Hausmann, W., *Z. physiol. Chem.*, 1899, **27**, 95.

2 Van Slyke, D. D., *J. Biol. Chem.*, 1911-12, **10**, 15.

3 Ikeda, K., and Suzuki, S., U. S. Patent No. 1,015,891, Jan. 30, 1912.

4 Foster, G. L., and Schmidt, C. L. A., PROC. SOC. EXP. BIOL. AND MED., 1921-22, **19**, 348; *J. Biol. Chem.*, 1923, **56**, 545; *J. Am. Chem. Soc.*, 1926, **48**, 1709.

5 Kuhn, R., and Desnuelle, P., *Ber. deut. chem. Gesel.*, 1937, **70**, 1907.

6 Albanese, A. A., *J. Biol. Chem.*, 1940, **134**, 467.

7 Csonka, F. A., *Cereal Chem.*, 1941, **18**, 523.

8 Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, 1941, **35**, 1369.

9 Whitehorn, J. C., *J. Biol. Chem.*, 1923, **56**, 751.

10 Dubnoff, J. W., *J. Biol. Chem.*, 1941, **141**, 711.

a protein hydrolysate for subsequent estimation by the Sakaguchi reaction. The necessity of removing the basic amino acids from the zeolites with strong neutral salt (NaCl, KCl) solutions, largely precludes this procedure as a preliminary step for their further use.

Recently Turba[11] has used chromatographic adsorption on activated fuller's earths (Filtrol-neutrol and Floridin XXF extra) to separate arginine, histidine, and lysine from each other and from the monoamino acids. M/6 KH_2PO_4 and a mixture of N H_2SO_4 and pyridine (5:1) are used as the eluting agents. No report has been seen on the application of this procedure to protein hydrolysates.

The introduction by Adams and Holmes[12] of two types of ion exchange resins, one capable of fixing mineral and strong organic acids and the other able to bind cations, opened the possibility of separating amino acids from the non-nitrogenous compounds in protein hydrolysates. The resins, Amberlites IR-1, IR-100 and IR-14 (Resinous Products and Chemical Company) have proven to be especially valuable for the separation of the basic amino acids from the other constituents of protein hydrolysates. A concentrate is thus obtained which is very suitable for the isolation and large scale preparation of arginine, histidine, and lysine.

The following experiment is representative: 11.7 g of commercial blood fibrin (N 16.0%) were hydrolyzed with 200 cc of 1:1 HCl for 20 hr. The excess acid was removed by concentration *in vacuo* and the residue was taken up in 500 cc of hot water, decolorized with 2 g of activated carbon, and filtered. The residue was thoroughly washed. The combined filtrates were concentrated to dryness. The residue was taken up in 800 cc of water and stirred with the acid binding resin, IR-4, until the reaction of the solution was approximately pH 6. The resin was removed and thoroughly washed. The amino acid solution (volume 1500 cc) was passed through a 10″ x ¾″ column of cation adsorption resin, IR-100, (hydrogen cycle) at the rate of 40 to 50 cc per min. The column was washed with water and the basic amino acids were removed by exchange with 7% HCl. The completion of the removal was followed by testing the effluent with phosphotungstic acid and by the Sakaguchi reaction. The effluent solution was concentrated to remove the excess HCl and diluted to 100 cc. Total nitrogen, 538 mg; nitrogen *not* precipitated by phosphotungstic acid, 30 mg. Tests for cystine, tyrosine, proline, and hydroxyproline were negative. The basic amino acids were isolated from a 50 cc aliquot of this solution according to the modified Kossel procedure. Histidine was precipitated with Ag_2SO_4 and $Ba(OH)_2$ at pH 7.4 and isolated as the nitranilate. Arginine was precipitated with Ag_2SO_4 and $Ba(OH)_2$ in strongly alkaline solution and isolated as the flavianate. 100 cc of alcohol were added to the filtrate from the arginine silver precipitate and the solution was concentrated at low temperature to remove all the ammonia. Lysine was isolated as the picrate without phosphotungstic acid precipitation. Yields: arginine, 6.4%; histidine, 2.0%; and lysine, 7.5%. Electrolytical transport purification[6] of a fibrin hydrolysate yielded 7.0% of arginine, 2.2% of histidine, and 6.0% of lysine, calculated on the basis of 16.0% of nitrogen in the protein.

Summary. A method is described for the separation of ammonia, arginine, histidine, and lysine from the other constituents of protein hydrolysates, based on the use of synthetic ion exchange resins. The procedure is especially valuable for the preparation of concentrates of the basic amino acids.

[11] Turba, F., *Ber. deut. chem. Gesel.*, 1941, **74B**, 1829, from *Chem. Abs.*, 1942, **36**, 5494.

[12] Adams, B. A., and Holmes, E. L., *J. Soc. Chem. Ind.*, 1935, **54**, 1.

13923

Effect of Alkyl-Dimethyl-Benzyl-Ammonium Chlorides (Zephiran) Upon Tetanus Toxin.

ERWIN NETER.

From the Laboratories of the Children's Hospital and the Department of Bacteriology and Immunology, University of Buffalo, School of Medicine.

Numerous organic and inorganic compounds destroy the toxic properties of bacterial exotoxins. An excellent review on this subject was presented by Eaton.[1] Certain substances reduce the toxicity without affecting the antigenicity of the toxins, whereas others destroy both of these properties. An investigation[2] on the possible detoxifying effects of antimicrobial substances of biological origin revealed that some of them, such as pyocyanase, detoxify tetanus toxin and others, such as tyrothricin and actinomycin A, fail to do so. In the following communication are presented the results of experiments on the action upon tetanus toxin of Zephiran chloride, a mixture of high molecular alkyl-dimethyl-benzyl-ammonium chlorides. This substance contains alkyl radicals ranging from C_8H_{17} to $C_{13}H_{37}$ whose source is a mixture of fatty acids of cocoanut oil.

Material and Methods. Tetanus toxin was obtained through the courtesy of Dr. Edwin F. Voigt, Director, Human Biological Division, Lederle Laboratories. This toxin, in a dilution of 1:1,000 (volume 0.2 ml), when injected subcutaneously into the hind leg of white mice, caused local tetanus within 18 hr and death within 24 to 48 hr. In a dilution of 1:10,000 it produced local tetanus after 24 to 48 hr and usually death after several days. In a dilution of 1:100,000 it caused no noticeable toxic effects.

An aqueous solution of Zephiran chloride was used. According to Mr. Charles G. Marshall, Alba Pharmaceutical Company, Inc., New York, New York (personal communication) this solution has a pH of approximately 7.1.

White mice weighing between 18 and 22 g were used.

Results. In the first experiment the effects of Zephiran chloride upon tetanus toxin in various concentrations was studied. The final concentration of Zephiran chloride was 1:2,000 and those of tetanus toxin 1:100, 1:1,000, and 1:10,000, respectively. The mixtures (volume 0.2 ml), immediately after they were prepared, were injected subcutaneously into the hind leg of mice. The animals were observed daily for 14 days for the appearance of local tetanus. The results of this experiment are presented in Table I.

It may be seen from Table I that Zephiran chloride (1:2,000) completely prevented the toxic effects of tetanus toxin in dilutions of 1:10,000 and 1:1,000. Tetanus toxin in a dilution of 1:100 mixed with Zephiran chloride (1:2,000) produced local tetanus and caused death after 14 days; however, Zephiran chloride markedly delayed the lethal effect of tetanus toxin. Essentially the same results were obtained in repeated experiments. With this particular toxin at least 10 minimal lethal doses were detoxified by 0.1 mg of Zephiran chloride. It may be mentioned that some of the mice injected with a mixture of tetanus toxin and Zephiran chloride died several days or even 2 weeks after the injection without having developed any signs of local or generalized tetanus.

A second series of experiments was carried out to determine the smallest amount of Zephiran chloride capable of abolishing the toxic effects of tetanus toxin. It was found that Zephiran chloride in a dilution of 1:2,000 completely abolished the toxic effects of 10 minimal lethal doses of tetanus toxin in mice, but in dilutions of 1:20,000 and higher it failed to affect its activity.

In order to determine whether or not this effect of Zephiran chloride upon tetanus toxin is due to the pH of the Zephiran chloride solution, the following experiments

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1 Eaton, M. D., *Bact. Rev.*, 1938, **2**, 3.

2 Neter, E., *Science*, 1942, **96**, 209.

TABLE I.
Effect of Alkyl-dimethyl-benzyl-ammonium Chlorides (Zephiran) upon Tetanus Toxin Injected into Mice.

Days of observation	Zephiran (1/2000) plus tetanus toxin in dilutions of			Physiological salt solution plus tetanus toxin in dilutions of		
	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^2$	$1/10^3$	$1/10^4$
1	—	—	—	D	++++	—
2	+	—	—		D	++++
3	++++	—	—			++++
4	++++	—	—			++++
5	++++	—	—			++++
8	++++	—	—			++++
10	++++	—	—			D
14	D	—	—			

— = No signs of local tetanus.
+ to ++++ = Various degrees of local tetanus.
D = Death.

were performed. Zephiran chloride was dissolved in disodium phosphate-citric acid buffer solution of pH 7.0 with phenol red as indicator. The resulting pH ranged from approximately 7.0 to approximately 7.4, depending upon the concentration of Zephiran chloride. Controls revealed that tetanus toxin dissolved in buffer solutions of corresponding pH (7.0 to 7.4) produced toxic effects in mice. No marked difference in the time of appearance and severity of local tetanus were noted at this pH range. Furthermore, the experiments revealed that Zephiran chloride markedly reduced the toxic effects of tetanus toxin in buffer solutions of pH 7.0 to 7.4. It may be concluded, therefore, that under the conditions of the experiment the effects of Zephiran chloride upon tetanus toxin are independent of any changes due to influences of the pH of the solution.

Tissue fluids interfere with the activity of many antimicrobial agents. It is of interest, therefore, to see whether serum prevents the detoxifying effects of Zephiran chloride. Human serum (0.1 ml) was mixed with Zephiran chloride prior to the addition of tetanus toxin. This mixture caused local tetanus and death of the mice, indicating that serum inhibited the detoxifying action of Zephiran chloride. Quantitative experiments showed that human serum in dilutions up to 1:100 almost completely prevented the toxin inhibiting activity of Zephiran chloride. Serum in a dilution of 1:1,000 failed to do so. On the other hand, it is interesting to note that human serum and Zephiran chloride did not interfere to any great extent with the detoxifying effects of this compound. The results of this experiment indicate, furthermore, that Zephiran chloride rather rapidly detoxifies tetanus toxin and that this change is not readily reversible.

Discussion. High molecular alkyl-dimethyl-benzyl-ammonium chlorides (Zephiran chloride) possess marked germicidal and bacteriostatic potency. This antimicrobial agent also prevents the toxic effects of tetanus toxin in mice. Ten minimal lethal doses of tetanus toxin were detoxified by 0.1 mg of Zephiran chloride. It has not as yet been determined whether Zephiran chloride interferes solely with the toxic properties of the toxin or affects also its antigenic pattern. Furthermore, the mode of action of this substance upon tetanus toxin remains to be elucidated, particularly in regard to the question whether it causes strictly chemical changes or primarily physical effects. Formaldehyde, for instance, detoxifies by chemical action, whereas lanolin probably acts on the basis of adsorption on colloidal particles (Ramon and associates).[3]

It is of interest to mention that the salts of fatty acids, beginning with those containing 8 carbon atoms in the chain, cause a de-

[3] Ramon, G., Lematayer, E., Richou, R., and Nicol, L., *Rev. Immunol.*, 1937, **3**, 285.

struction of diphtheria toxin. Larson and associates[4-6] found that the sodium salt of ricinoleic acid destroys the toxic properties of diphtheria toxin and Schmidt[7] reported that this substance changes its antigenic properties as well. According to Schmidt[7] not all unsaturated fatty acids destroy bacterial exotoxins; for instance, fumaric acid and maleic acid do not have this effect.

From a practical point of view it seems desirable to have available for the prevention or treatment of infections caused by toxin-producing bacteria antimicrobial compounds of marked effectiveness and low toxicity which, moreover, may be destructive to toxins. Zephiran chloride in concentrations which are bacteriostatic or bactericidal *in vitro* and *in vivo* inhibits the toxic effects of tetanus toxin. The inactivation of this toxin takes place almost immediately. However, it must be emphasized that presence of serum definitely interferes with this inactivation. The possible effects of antimicrobial agents upon the activity of toxins deserves further study.

Summary. (1) Alkyl-dimethyl-benzyl-ammonium chlorides (Zephiran chloride) in a concentration of 1:2,000 inactivates tetanus toxin. This effect takes place almost immediately. Zephiran chloride in a dilution of 1:20,000 fails to do so. (2) Human serum mixed with Zephiran chloride prior to the addition of tetanus toxin markedly interferes with the inactivation of toxin by this compound. Addition of serum to a mixture of Zephiran chloride and tetanus toxin does not reactivate the toxin.

4 Larson, W. P., and Nelson, E., PROC. SOC. EXP. BIOL. AND MED., 1923-24, **21**, 278.

5 Larson, W. P., Evans, R. D., and Nelson, E., PROC. SOC. EXP. BIOL. AND MED., 1924-25, **22**, 194.

6 Larson, W. P., and Halvorson, H. O., PROC. SOC. EXP. BIOL. AND MED., 1924-25, **22**, 550.

7 Schmidt, S., *Bioch. Z.*, 1932, **256**, 158.

13924

Effects of Alkyl-Dimethyl-Benzyl-Ammonium Chlorides upon Plasma-Coagulation by Staphylococcus and Fibrinolysis by Streptococcus.

ERWIN NETER.

From the Laboratories of the Children's Hospital and the Department of Bacteriology and Immunology, University of Buffalo, School of Medicine.

Certain surgical antiseptics and chemotherapeutic substances inhibit *in vitro* streptococcal fibrinolysis and coagulation of plasma by staphylococcus. Madison and Snow[1] showed that tincture of iodine (1:3,000) renders fibrin but one-eighth its normal susceptibility to liquefaction by streptococci. Other substances such as merthiolate (1%), phenol (2%), and mercuric chloride (1%) did not affect fibrinolysis, nor did sulfanilamide (10%) and prontosil (2.5%). Spink and Vivino[2] observed that sulfadiazine inhibits the plasma-clotting effect of staphylocoagulase; in contrast, sulfathiazole and, to a slightly lesser extent, sulfanilamide accelerated clot formation. Recently it was shown[3] that two antimicrobial agents of biological origin, namely, tyrothricin and actinomycin A, inhibit both coagulation of plasma by staphylococcus and lysis of plasma clots by group A *beta hemolytic streptococcus*. Alkyl-dimethyl-benzyl-ammonium chlorides (Zephiran chloride), a disinfectant of marked antimicrobial activity, inhibits the toxic effects of tetanus toxin.[4] It seemed of interest to determine what

1 Madison, R. R., and Snow, J. E., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 592.

2 Spink, W. W., and Vivino, J. J., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 37.

3 Neter, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 163, 167.

4 Neter, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 254.

effects, if any, this compound has upon plasma-coagulation by staphylococcus and fibrinolysis by streptococcus.

Material and Methods. An aqueous solution of alkyl-dimethyl-benzyl-ammonium chlorides (Zephiran chloride) was used in the experiments. In the tests on fibrinolysis the technic of Tillett and Garner[5] was followed. A strain of *beta hemolytic streptococcus* (Group A) obtained from man was used as a source of fibrinolysin. The technic of the staphylococcal coagulase test was essentially the same as that described by Fisk.[6] A strain of *Staphylococcus aureus hemolyticus* obtained from a skin lesion was employed as a source of coagulase.

TABLE I.

Effect of Alkyl-dimethyl-benzyl ammonium Chlorides (Zephiran) upon Plasma-Coagulation by Staphylococcus.

Time of observation hr	0.1 ml of Staphylococcus culture 0.9 ml of Zephiran in dilutions of 1/10⁴	1/10⁵	1/10⁶	0
1	—	—	++++	++++
5	—	++++	++++	++++
18	—	++++	++++	++++

Results read after the addition of 0.2 ml of oxalated human plasma.
— = No clot formation.
++++ = Complete clot formation.

Plasma was obtained from human sources. One ml of a 2% potassium oxalate solution was evaporated in small bottles. Five ml of human blood were added and shaken well. Plasma was removed following centrifugalization of the blood.

Results. In the first experiment, the action of alkyl-dimethyl-benzyl-ammonium-chlorides (Zephiran chloride) in various concentrations upon plasma-coagulation by staphylococcus was determined. To 0.1 ml of an 18-hr infusion broth culture of staphylococcus were added, respectively, 0.9 ml of Zephiran chloride in dilutions of 1:10,000, 1:100,000, 1:1,000,000, and 0.9 ml of physiological salt solution as control. Then, 0.2 ml of oxalated human plasma was placed in each tube. The tubes were incubated at 37°C. Clot formation was noted at various intervals. The results of this experiment are summarized in Table I.

It may be seen from Table I that Zephiran chloride in a dilution of 1:10,000 completely prevented clotting of human plasma by staphylococcus for 18 hr. In a dilution of 1:100,000 it caused a delay in the clot-formation. Essentially the same results were obtained in several experiments. In some tests, Zephiran chloride even in a dilution of 1:1,000,000 caused a transitory inhibition of plasma-coagulation by staphylococcus. The question arises as to whether this inhibitory action is due to effects upon the staphylococcal coagulase or upon the plasma itself. Zephiran chloride in a dilution of 1:1,000 inhibits the clotting of oxalated plasma after the addition of minimal amounts of calcium chloride; in dilutions of 1:100,000 and above, however, its anticoagulating effects are minimal. Furthermore, it was found that the addition of calcium chloride or rabbit clotting globulin* to a mixture of staphylococcal culture, Zephiran chloride, and plasma caused clot-formation. Although these experiments do not allow of any final conclusions as to the mode of action of Zephiran chloride upon plasma-coagulation by staphylococcus, nevertheless, they seem to indicate that the inhibitory effect of this compound is not directed against the mechanism of clot formation and therefore by exclusion may be due to its action upon the staphylococcus culture or the staphylocoagulase.

A second series of experiments was carried out to determine the effects of Zephiran chloride upon fibrinolysis by *beta hemolytic streptococcus*. The experiment was set up as follows: To 0.1 ml of an 18-hr infusion broth culture of *streptococcus hemolyticus* was added, respectively, 0.9 ml of Zephiran chloride in dilutions of 1:10,000, 1:100,000, 1:1,000,000 and 0.9 ml of physiological salt solution. As control, 0.1 ml of infusion broth was mixed with 0.9 ml of Zephiran chloride in a dilution of 1:10,000. Follow-

[5] Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485.

[6] Fisk, A., *Brit. J. Exp. Path.*, 1940, **21**, 311.

* The rabbit-clotting globulin was obtained from Lederle Laboratories through the courtesy of Dr. A. L. Joyner.

TABLE II.
Effect of Alkyl-dimethyl-benzyl-ammonium Chlorides (Zephiran) upon Fibrinolysis by *Hemolytic Streptococcus.*

Time of observation	0.1 ml of Streptococcus culture 0.9 ml of Zephiran in dilutions of 1/10⁴	1/10⁵	1/10⁶	0	0.1 ml broth 0.9 ml Zephiran (1/10,000)
15 min	—	++++	++++	++++	—
30 "	—	++++	++++	++++	—
2 hr	++	++++	++++	++++	—
3 "	++++	++++	+	—	+
5 "	++++	++++	—	—	++++
18 "	++++	—	—	—	++++

Results read after the addition of 0.2 ml of oxalated human plasma and 0.35 ml of 0.25% $CaCl_2$.
— = No clot formation.
+ to ++++ = Various degrees of clot formation.

ing incubation for 90 min at 37°C, 0.2 ml of oxalated human plasma and 0.35 ml of 0.25% calcium chloride solution were added to all tubes. The tubes were incubated at 37°C. The results are presented in Table II.

Table II reveals that Zephiran chloride in a dilution of 1:10,000 prevented completely the lysis of fibrin clots by streptococcus. A marked delay in fibrinolysis resulted from the presence of Zephiran chloride in a dilution of 1:100,000. It is also evident that Zephiran chloride in a dilution of 1:10,000 caused a retardation in the clot-formation following the addition of calcium chloride solution. In other experiments this effect was less pronounced.

In order to determine whether or not inhibition of fibrinolysis necessitates incubation of Zephiran chloride and streptococcus culture, the following experiment was carried out. In part I, Zephiran chloride was incubated with the streptococcus broth culture for 1 hr at 37°C prior to the addition of plasma and calcium chloride solution; in part II, Zephiran chloride, streptococcus culture, plasma, and chloride solution were mixed without previous incubation. The experiment revealed that in part I Zephiran chloride in dilutions up to 1:160,000 inhibited fibrinolysis, whereas in part II Zephiran chloride in dilutions up to only 1:40,000 caused this effect. Similar results were obtained in repeated experiments. It may be concluded that the action of Zephiran chloride upon plasma alone does not account for the above described effect upon streptococcal fibrinolysis. The significance of the inhibitory effects of Zephiran chloride upon plasma-coagulation by staphylococcus and fibrinolysis by *hemolytic streptococcus* and their possible applications, remain to be determined.

Summary. (1) Zephiran chloride in a dilution of 1:10,000 inhibits and in higher dilutions (up to 1:1,000,000) delays the clotting of oxalated human plasma by staphylococcal cultures. (2) Zephiran chloride in dilutions up to 1:100,000 inhibits fibrinolysis by *hemolytic streptococcus.*

13925 P

Transformation of Virus of Rabbit Fibroma (Shope) into that of Infectious Myxomatosis (Sanarelli).

RALPH B. HOULIHAN. (Introduced by L. W. Parr.)

From the Department of Preventive Medicine, University of Virginia, School of Medicine, Charlottesville, Va.

Berry,[1] Hoffstadt,[2] and Hurst[3] have reported the transformation of fibroma virus into myxoma virus employing crude tissue suspensions of myxoma virus. Meck[4] and Hyde[5] were unable to confirm these observations. Recently Gardner and Hyde[6] have accomplished the transformation with 3 of 7 elementary body suspensions of myxoma virus. Hurst[3] has obtained positive results in 5 of 7 rabbits and Hoffstadt[2] in 7 of 20. The purpose of the experiments here reported was to amplify these observations and to study factors which might account for the inconsistent results.

A 10% suspension of a dermal tumor of virulent myxoma virus was heated for 35 min at 60°C in a water-bath, then kept in sealed ampoules at 4°C until used. This tissue suspension of heat-inactivated myxoma virus was submitted to the following procedures:

1. Serial testicular passage.
2. Mixture with (a) active fibroma virus, (b) attenuated fibroma virus, (c) agents producing reduction, inflammation, increase in virulence; and subsequent testicular injection or serial passage of these mixtures.

Serial testicular passage was performed by removing the inoculated testicle 7 days after injection. This testicular tissue was triturated; portions of a 10% suspension were mixed with heat-inactivated myxoma virus and injected into the testicle of another rabbit.

Two sets of experiments were performed at separate times with freshly prepared heat-inactivated myxoma virus. New Zealand rabbits weighing approximately 4½ lb were used throughout the experiments, and they were kept in isolated cages to prevent possible cross-infection. Temperature readings and observations were made daily.

Experiment I.

Group 1. 1 cc of heat-inactivated myxoma virus passed serially through 3 rabbits.

Group 2. 1 cc of heat-inactivated myxoma virus plus 0.5 cc of active fibroma virus passed serially through 3 rabbits.

Group 3. 1 cc of heat-inactivated myxoma virus plus 0.5 cc of active fibroma virus passed serially through 2 rabbits.

Group 4. Control group. Dermal and testicular tumors of active myxoma virus and active fibroma virus injected into normal rabbits.

Experiment II.

Group 1. 1 cc of heat-inactivated myxoma virus passed serially through 2 rabbits.

Group 2. 1 cc of heat-inactivated myxoma virus plus 0.5 cc of attenuated fibroma virus (long storage in glycerol, causing minimal lesions) injected into 4 rabbits.

Group 3. 1 cc of heat-inactivated myxoma virus plus 0.5 cc of active fibroma virus passed serially through 2 sets of 4 rabbits each.

Group 4. 1 cc of heat-inactivated myxoma virus plus 0.07 cc of active fibroma virus injected into 4 rabbits.

Group 5. 1 cc of heat-inactivated myxoma virus plus the following materials: (Each mixture was injected into 1 rabbit, except 2(a) which was tested in 5 rabbits.)

1. Reducing agents.
   a. 1 cc of 5% $CaCl_2$.
   b. 0.02 cc of 1:50 cysteine hydrochloride.
   c. 0.1 cc of 2% sodium thioglycolate.
2. Inflammatory agents.
   a. 0.5-1 cc of beef extract and aleuronat in olive oil.

[1] Berry, G. P., and Dedrick, H. M., *J. Bact.*, 1936, **31**, 50.

[2] Hoffstadt, R. E., and Pilcher, K. S., *J. Inf. Dis.*, 1941, **68**, 67.

[3] Hurst, E. W., *Brit. J. Exp. Path.*, 1937, **18**, 23.

[4] Meck, J. S., and Acree, E. G., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 387.

[5] Hyde, R. R., *Am. J. Hyg.*, 1936, **24**, 217.

[6] Gardner, R. E., and Hyde, R. R., *J. Inf. Dis.*, 1942, **71**, 47.

b. 1 cc of 24-hr broth culture of *E. coli*.
c. 1 cc of 10% suspension of vaccinia virus.

3. Virulence-enhancing agent.
a. 2 cc of 5% gastric mucin.

Group 6. Control group. Dermal and testicular tumors of active myxoma virus and active fibroma virus injected into normal rabbits.

Results. The Berry transformation was successful only in Group 3 of Experiment I. Rabbit 1 exhibited generalized myxomatosis on the 12th day after injection and subsequently recovered. Testicular tissue removed from this rabbit on the 7th day following injection produced typical fatal myxomatosis in two normal rabbits. The possibility of accidental infection of this transformation rabbit was eliminated because there were no myxomatous rabbits in the laboratory at the time.

Typical fibromatosis and myxomatosis were produced in the control group.

Attempts to facilitate the transformation by the use of attenuated fibroma virus and small doses of active fibroma virus were not successful.

Heat-inactivated myxoma virus was not reactivated by inflammatory, reducing, and virulence-enhancing agents, or by serial passage.

Discussion. The occurrence of the Berry transformation in only one of 21 rabbits emphasizes the observations of other workers that this transformation is not accomplished easily.[1,2,3,5,6] Our inability to reactivate heat-inactivated myxoma virus with diverse agents amplifies the evidence of Berry[7,8] and Gardner and Hyde.[6] Additional data are required to prove whether fibroma virus reactivates heat-inactivated myxoma virus or is actually transformed into active myxoma virus.

7 Personal communication.

8 Berry, G. P., and Dedrick, H. M., *J. Bact.*, 1936, **32**, 356.

13926

Initiation of Secretory Changes in Transplanted Mammary Adenocarcinoma of the Rat by Pituitary Lactogenic Hormone.*

MILTON J. EISEN. (Introduced by W. H. Woglom.)

From the Department of Cancer Research, College of Physicians and Surgeons, Columbia University, New York City.

Secretory phenomena in a transplanted mammary adenocarcinoma (R2426), maintained in inbred August rats, have been described following treatment of hosts of either sex with 0.166 mg estradiol benzoate or 0.2 mg estradiol dipropionate twice weekly.[1] Subsequent experiments have demonstrated that daily injections of considerably smaller quantities of the hormones are equally effective in initiating these changes, which varied in degree from isolated foci of secretory vacuoles in the neoplastic cells, evident only on microscopic examination, to extensive transformation of tumors into lactating cystic masses containing thick milk-like fluid. Neoplasms of the latter type bore little resemblance to the original adenocarcinoma. Tumors were not influenced by the normal hormonal variation incident upon age or sex,[2] nor did castration, pregnancy and lactation, or testosterone propionate affect either their growth or their histologic characteristics.[1]

Experimental. It appeared of interest to ascertain the influence of the lactogenic

* Anterior pituitary lactogenic hormone was generously furnished by the Research Department of Parke, Davis & Co., Detroit, Mich.; progesterone and estradiol dipropionate by Ciba Pharmaceutical Products, Inc., Summit, N.J.

1 Eisen, M. J., *Cancer Research*, 1941, **1**, 457.

2 Eisen, M. J., *Am. J. Cancer*, 1940, **39**, 36.

TABLE I.
Effect of Anterior Pituitary Lactogenic Hormone on Transplanted Mammary Adenocarcinoma (R 2426) in Nursing Rats.

Rat	Age of animals when tumor trans-planted, days	Littered, days after trans-plantation	No. nursed	No. days nursing when treated	Treatment	Killed, days after trans-plantation	Result
1	80	40	5	7	4 x 40 units	52	Secretory foci
2	80	47	8	12	6 x 40 "	66	" "
3	185	28	4	3	6 x 80 "	38	" "
4	213	15	3	10	5 x 80 "	31	No change

TABLE II.
Effect of Anterior Pituitary Lactogenic Hormone, Estradiol and Progesterone on Transplanted Mammary Adenocarcinoma (R 2426).

No. of rats F	M	Status	Previous treatment	Lactogenic hormone	Result
2	2	Intact	None	4 x 40 units	No secretion
2	2	"	Progesterone	"	" "
6	6	Castrated	"	"	" "
2	2	Intact	Estradiol, Progesterone	"	Secretory foci
3	5	"	" "	None	" "

factor of the pituitary gland on a mammary tumor of this type which reacts so strikingly to the influence of exogenous estrogen. The biologic characteristics of this lactogenic hormone have been reviewed in detail by Riddle.[3] When employed clinically in cases of deficient lactation the results have proved controversial, some investigators[4,5] having recorded a favorable influence, while others[6] have denied any specific action.

The possibility that it might have some action on this carcinoma was tested in 36 tumor animals: 4 suckling females (Table I) and 32 other rats, including non-suckling females, normal males, both normal and castrated females and males following previous treatment with progesterone, and intact animals of either sex treated earlier with estradiol and progesterone (Table II). The effects of estradiol and progesterone alone were also examined.

The suckling rats bore subcutaneous tumors in the right flank and had successfully cared for their litters for 3 to 12 days. Suckling was permitted to continue during the period of treatment. From one-half to one cc (40 to 80 crop gland units) was injected subcutaneously at a distance from the tumors daily for from 4 to 6 days and the animals were sacrificed 48 hr after the last treatment. Evidence of secretory activity was found in the tumors of 3 among 4 of these rats. The single unaffected tumor had been transplanted after the onset of pregnancy. Whether the sensitivity of the others was related to the longer and more complete normal hormonal stimulation to which they had been subjected from the beginning of pregnancy cannot be stated definitely on the basis of the available data.

The alterations could not be perceived with certainty upon gross examination, but under the microscope they were demonstrable in many areas throughout the tumors. In the altered zones some or all the cells of a considerable number of alveoli contained distinct secretory vacuoles in the area bordering the acinar cavities. These functioning neo-

[3] Riddle, O., *J. Am. Med. Assn.*, 1940, **115**, 2276.

[4] Kurzrok, R., Bates, R. W., Riddle, O., and Miller, E. G., Jr., *Endocrinology*, 1934, **18**, 18.

[5] Ross, J. R., *Endocrinology*, 1938, **22**, 429.

[6] Stewart, H. L., Jr., and Pratt, J. P., *Endocrinology*, 1939, **25**, 347.

plastic acini were more regular and rounded in shape than those in the unchanged tumor zones.

The treatment exerted no measurable effect upon the amount of milk secreted by the mamma, nor did histologic examination disclose any departure from the manifestations normally occurring in lactating mammary tissue.

As for the non-suckling and the male animals, pituitary lactogenic hormone in 4 daily doses of 40 units each, administered to 2 females and 2 males bearing transplanted tumors of 42 days, produced no changes in the neoplasms. Likewise similar treatment of 6 spayed females and an equal number of castrated males following 15 injections of 0.25 mg each of progesterone dissolved in sesame oil, or of 2 intact females and 2 males that had received 17 injections of progesterone, exerted no influence on the transplanted cancers. The progesterone, given 3 times weekly, was begun 10 days after transplantation, the lactogenic factor 3 days following the final treatment with the former. Tumors of animals that received only progesterone failed to show changes.

While the administration to 3 normal females and 5 males of 17 injections, given 3 times weekly, of 0.25 mg of progesterone and 0.025 mg of estradiol dipropionate resulted in extensive secretion in the tumors, the alterations did not differ from those induced by this quantity of estradiol alone. Treatment of 2 normal females and an equal number of males with 4 daily doses of 40 units each of pituitary lactogenic hormone, begun 3 days following the completion of the previously described course of progesterone and estradiol, did not enhance the effects of the latter combination of hormones.

Summary. The daily injection of 40 to 80 crop gland units of pituitary lactogenic hormone for 4 to 6 days into rats suckling their young induced secretory changes in a transplanted mammary adenocarcinoma. The hormone produced no effect on the tumors of non-nursing females or normal males. Progesterone also exerted no influence, nor did it affect, when given simultaneously with estradiol dipropionate, the secretory phenomena induced in the neoplastic cells by the latter substance alone. Pituitary lactogenic hormone failed to induce secretion in the tumor cells of animals previously treated with progesterone, and did not enhance the alterations produced by the combined administration of progesterone and estradiol.

13927

Absorption and Excretion of Sulfamethyldiazine (2-Sulfanilamido-4-methyl-pyrimidine) in Human Subjects.

ROBERT A. GOODWIN, JR., OSLER L. PETERSON AND MAXWELL FINLAND.

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston.

Sulfamethyldiazine (2 - sulfanilamido - 4-methyl-pyrimidine) was prepared and characterized by Roblin *et al.*[1] at the same time that they reported on sulfadiazine. Both these drugs were found to be active in mice against experimental pneumococcal and streptococcal infections in mice. The former was found to be 2½ times as soluble as the latter in water at 37°C and its N4-acetyl derivative was also reported to be twice as soluble as that of sulfadiazine. Maximum blood levels in mice of sulfamethyldiazine were found to be slightly higher than those obtained on the same dose of sulfadiazine. The 4-6-dimethyl derivative[2] is considerably

[1] Roblin, R. O., Jr., Williams, J. H., Winnek, P. S., and English, J. P., *J. Am. Chem. Soc.*, 1940, **62**, 2002.

[2] Roblin, R. O., Jr., Winnek, P. S., and English, J. P., *Ibid.*, 1942, **64**, 567.

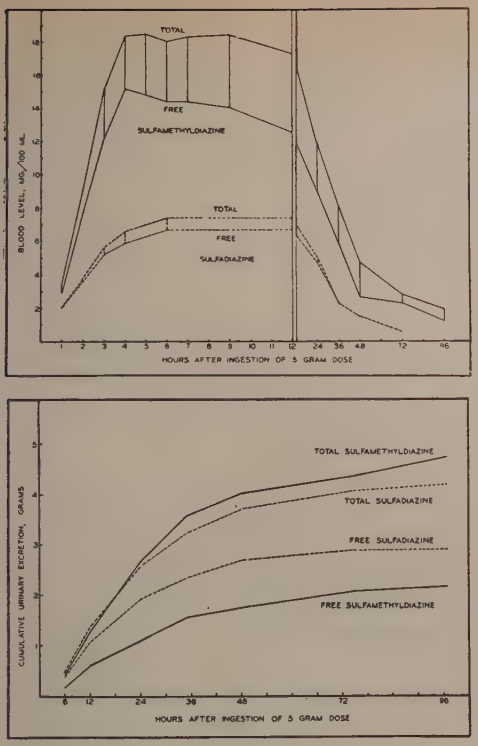

FIG. 1.

Comparison of average values obtained in 3 subjects receiving 5 g of sulfamethyldiazine orally 1 hour after breakfast with those obtained in 11 subjects receiving 5 g of sulfadiazine in the same manner. Each dose was given with 500 ml of water and the daily fluid intake was maintained at about 3 liters.

Above. Average blood levels.

Below. Average cumulative urinary excretion.

more soluble than the monomethyl compound. Numerous reports are available on the use of sulfadiazine and recently reports have appeared in England concerning clinical trials of the dimethyl derivative.[3,4] The present report deals with preliminary studies on the pharmacology of sulfamethyldiazine* in humans and some observations on the antipneumococcal action of this compound. The methods used were the same as those employed in similar studies reported from this laboratory.[5-7]

Blood levels. A single 5 g dose of sulfamethyldiazine was administered to each subject with about 500 ml of water about an hour after breakfast and blood and urine samples were tested as in previous studies[7] except that more frequent blood samples were taken during the first 12 hr. The average blood levels in 3 subjects are shown in Fig. 1 which also shows the average blood levels in 11 subjects who received sulfadiazine in the same manner. The concentration of sulfamethyldiazine in the blood rose more rapidly, attained levels about twice as high, and were sustained for much longer periods than sulfadiazine. Both the total amount and the proportion of the former compound that was found in the conjugated form were greater than for sulfadiazine. The maximum blood levels (Table I) averaged over 20 mg per 100 ml and occurred in from 4 to 9 hr; of this amount about 20% was determined as the conjugated (? acetylated) compounds. After 24 hr, the blood levels of sulfamethyldiazine were from 10 to 14 mg per 100 ml, of which from 2 to 3 mg were conjugated. This compares with an average of 5 mg per 100 ml 24 hr after the sulfadiazine dose, with only small amounts found in the conjugated form. After 72 hr there was still from 1 to 3.5 mg per 100 ml of sulfamethyldiazine in the blood. Most of the drug found in the blood, and sometimes all of it, was shown to be in the plasma (Table II).

Urinary excretion (Fig. 1, lower portion, and Table I). Almost all of the ingested sulfamethyldiazine (84-100%) was recovered from the urine. From 40 to 70% (average 53%) was recovered in the first 24 hr and from 80 to almost 100% (average 89%) was recovered in the first 72 hr. Of the drug excreted during the first 24 hr, from 51 to 61% was recovered as conjugated (? acetyl-

[3] McCartney, D. W., Smith, G. S., Luxton, R. W., Ramsay, W. A., and Goldman, J., *Lancet*, 1942, **1**, 639.

[4] Jennings, P. A., and Patterson, W. D., *Ibid.*, 1942, **2**, 308.

* The sulfonamide drugs used in these studies were supplied by the Lederle Laboratories, Inc.

[5] Strauss, E., Lowell, F. C., Taylor, F. H. L., and Finland, M., *Ann. Int. Med.*, 1941, **14**, 1360.

[6] Peterson, O. L., Strauss, E., Taylor, F. H. L., and Finland, M., *Am. J. Med. Sc.*, 1941, **201**, 357.

[7] Peterson, O. L., and Finland, M., *Ibid.*, 1942, **204**, 581.

TABLE I.
Maximum Blood and Urine Concentrations and Urinary Excretion After a Single 5 g Dose of Sulfamethyldiazine (2-sulfanilamido-4-methylpyrimidine).

Subject	Wt, kg	Max. blood conc., mg/100 ml			Max. urine conc., mg/100 ml			% administered drug recovered in urine		% recovered drug conjugated	
		Free	Total	Hr*	Free	Total	Period*	In 24 hr	In 72 hr	First 24 hr	24-72 hr
1	70	15.1	18.6	5	66	167	24-36	40.0	78.9	51.9	56.7
2	63	17.4	22.4	9	61	135	12-24	49.2	99.2	51.3	56.2
3	65	17.8	21.7	4	46	115	9-12	70.6	93.8	61.5	68.7

*Hours after ingestion of the dose.

TABLE II.
Distribution of Sulfamethyldiazine Between Plasma and Red Blood Cells.

Subject	Hr after dose	Concentration of Sulfamethyldiazine (mg/100 ml)						Hematocrit, %
		Whole blood		Plasma		Cells (calculated)		
		Free	Total	Free	Total	Free	Total	
2	36	8.0	10.2	15.5	19.0	0	0	44.0
	72	2.9	3.2	4.9	5.6	0.4 (?)	0.2	
3	4	12.1	14.7	17.8	21.7	4.3	5.3	43.0
	24	5.1	6.4	7.1	10.0	2.3 (?)	1.6	

ated) sulfamethyldiazine, and from 56 to 69% of the drug recovered during the next 48 hr were in the conjugated form. With sulfadiazine the average urinary recoveries were 52 and 81% during the first 24 and 72 hr respectively; and the proportion of conjugated drug averaged 26% and 35% during the first 24 hr and the next 48 hr, respectively. The maximum concentrations of sulfamethyldiazine in the urine varied from 115 mg per 100 ml in the nine to 12-hr sample in one subject to 167 mg per 100 ml in the 24 to 36-hr specimen in another. Only 44 mg of the former and 66 mg of the latter were determined as the free compound. Examination of the urinary sediments revealed no abnormal elements in any of the specimens except for rare red blood cells in one sample and occasional white blood cells in 3 samples, all obtained from the same patient who received sulfamethyldiazine.

Antipneumococcal activity of sulfamethyldiazine in human blood. This was studied with type III pneumococci by a method similar to that previously used in this laboratory.[8] The tests were carried out with defibrinated blood obtained 1 hr and 18 hr after ingestion of a 4 g dose. The blood levels were from 1.7 to 5.5 mg of free drug per 100 ml. The results are shown in Table III. At these low levels, 0.5 ml of the drug-containing blood exerted a marked bacteriostatic action on from 100 to 100,000 pneumococci.

Discussion. The higher blood levels obtained with sulfamethyldiazine suggest that therapeutic levels might be obtainable in human cases with much smaller doses than have been found necessary with sulfadiazine. The greater solubility also offers the possibility of less urinary tract irritation from precipitated crystals. In these respects the monomethyl compound should prove to be superior to the dimethyl derivative since the doses found necessary to maintain levels of the latter which are comparable to those obtained with sulfadiazine are usually twice those ordinarily employed.[3,4] However, the possibility of a deleterious action of sulfamethyldiazine on kidney structures, which is different from that which is attributable to the deposit of crystals of the drug, is suggested from chronic toxicity experiments in animals, and was not observed with sulfa-

[8] Spring, W. C., Jr., Lowell, F. C., and Finland, M., *J. Clin. Invest.*, 1940, **19**, 163.

TABLE III.

Antipneumococcal Action of Fresh Defibrinated Human Blood Obtained After Ingestion of a 4 g Dose of Sulfamethyldiazine.

Subject	Hr after dose	Blood level in mg/100 ml (free)	Hr. of incubation	Pneumococci added					
				10^6	10^5	10^4	10^3	10^2	10
H	0*	0	24	++	++	++	++	++	++
	1	1.7	24	++	++	++	+	—	—
			48	++	++	++	++	±	±
	19	3.8	24	++	—	—	—	—	—
			48	++	++	±	±	±	0
T	0	0	24	++	++	++	++	++	++
	1½	4.5	24	++	++	++	++	++	—
			48	++	++	++	++	++	±
	18	4.9	24	++	++	—	—	—	—
			48	++	±	±	±	±	0
M	0	0	24	++	++	++	++	++	0
	1	5.5	24	++	++	—	—	—	—
			48	++	++	±	±	±	±
	18	3.0	24	++	++	—	—	—	—
			48	++	++	±	±	±	0

0.5 cc defibrinated blood; type III pneumococcus in active growth phase; inoculum contained in 0.1 cc of broth; growth in sealed pyrex tubes rotated in incubator.

++ equals full growth with color change; + equals partial growth with slight color change; — equals no color change; ± equals no color change, few colonies in subcultures on blood agar.

*Control.

diazine.[9] The acute toxicities of sulfadiazine and sulfamethyldiazine, however, were approximately the same. Any clinical trial must, therefore, be undertaken with caution, particularly with respect to its use in patients requiring prolonged therapy. The possibility of neurological complications such as those encountered with sulfamethylthiazole must also be kept in mind.

Summary and conclusions. After a single 5 g oral dose of sulfamethyldiazine, the blood levels rise much more rapidly, higher levels are attained, and these are sustained longer than after a similar amount of sulfadiazine. The monomethyl compound was excreted more completely in the urine than sulfadiazine, but a larger proportion of the former was found in the conjugated (? acetylated) form. Sulfamethyldiazine warrants a clinical trial with doses that are smaller than those customarily used. Particular attention, however, should be paid to the possibility of renal or nerve damage in cases in which prolonged treatment with the drug is contemplated.

[9] Feinstone, W. H., Follis, R. H., Jr., and Williams, R. D., Sulfamethyldiazine (1 Sulfanilamido-4-methylpyrimine) as a chemotherapeutic agent. To be published.

13928

Oxidation of Hydrocarbons by Marine Bacteria.*

CARROLL W. GRANT† AND CLAUDE E. ZOBELL.

From the Scripps Institution of Oceanography, University of California, La Jolla.

Using oxygen consumption as a criterion it has been demonstrated that various kinds of pure and mixed hydrocarbons are oxidized by bacteria found in sea water and marine mud. The bacteria were grown in sea water enriched with hydrocarbons as the only source of energy. In the initial experiments the manometric technic was used for following oxygen consumption but later it was found that more accurate results were obtainable by determining the dissolved oxygen content of water.

Sea water inoculated with enrichment cultures was thoroughly shaken to insure uniformity in its composition and its saturation with oxygen, after which it was siphoned into 150 ml glass-stoppered bottles. While holding the bottles at an angle to provide for trapping the hydrocarbon in the shoulder of the bottle, 0.1 ml of hydrocarbon was introduced with a pipette. The bottle was then stoppered, care being taken not to entrap any air bubbles. Duplicate bottles of water were analyzed at once for oxygen content using the Winkler technic, and other bottles were analyzed after different periods of incubation in a water bath at 22°C.

The protocol of a representative experiment in which raw sea water was used is summarized in Table I. Similar results were obtained with aged sea water which was virtually organic matter-free as indicated by the fact that its B.O.D. was less than 1.0 mg/1.

It is especially noteworthy that toluene which is sometimes used as a preservative of biological materials was attacked by certain marine bacteria. Soil bacteria which oxidize toluene have been described by Gray and Thornton[1] and by Tauson.[2] Incidentally in these studies we found marine microörganisms which utilize phenol and cresol. Some of them tolerate a saturated aqueous solution of tricresol and as much as 1.0% phenol.

Besides the hydrocarbons listed in Table I, we have noted the bacterial oxidation of petroleum ether, paraffin wax, paraffin oil, vaseline, benzene, xylene, n-hexane, pyridine and naphthalene as indicated by oxygen consumption and by bacterial multiplication. Enough bacteria develop in aged sea water enriched with certain hydrocarbons to render it turbid. Pronounced differences have been noted in the rate of utilization of different kinds of hydrocarbons but due to differences in solubilities and other technical difficulties, many factors which influence utilizability of the various classes of hydrocarbons are still obscure. In general, it appears that aliphatic hydrocarbons are attacked more readily than cyclic or aromatic compounds, and long chains are more susceptible to bacterial oxidation than hydrocarbons of small molecular weight.

All 60 ml samples of sea water and all 0.1 g samples of mud examined show the presence of bacteria which are able to utilize hydrocarbons. From enrichment cultures several species of *Proactinomyces, Pseudomonas* and *Mycobacterium* have been isolated. Bacteria which oxidize various kinds of petroleum hydrocarbons have been isolated from soil by Tauson,[2] Haas *et al.*,[3] Stone *et al.*[4] and others. The marine hydrocarbon-oxidizing bacteria differ greatly in their ability to attack different hydrocarbons.

* Contribution from the Scripps Institution of Oceanography, New Series No. 182.

†Now at Brooklyn College, N.Y. Assisted by Grant No. 555 from the American Philosophical Society.

1 Gray, P. H. H., and Thornton, H. G., *Centralbl. f. Bakt.*, Abt. II, 1928, **73**, 74.

2 Tauson, V. O., *Planta*, 1929, **7**, 735.

3 Haas, H. F., Yantzi, M. F., and Bushnell, L. D., *Trans. Kansas Acad. Sci.*, 1941, **44**, 39.

4 Stone, R. W., Fenske, M. R., and White, A. G. C., *J. Bact.*, 1942, **44**, 169.

TABLE I.
Oxygen Consumed by Bacteria in Raw Sea Water Treated with Hydrocarbons.

Hydrocarbon	Initial dissolved O_2 content, mg/l	Dissolved O_2 after 14 days, mg/l	O_2 consumed 14 days, mg/l	O_2 consumed oxidizing hydrocarbon, mg/l
None (Control)	8.46	5.99	2.47	—
Crude oil	8.50	0.00	8.50	6.03
Petroleum ether	8.57	0.00	8.57	6.10
Kerosene	8.60	1.72	6.88	4.41
Gasoline	8.60	0.00	8.60	6.13
Toluene	8.64	0.00	8.64	6.17

They do not require hydrocarbons for their growth.

Marine hydrocarbon-oxidizing bacteria may influence the formation, accumulation and storage of petroleum or its products. Hydrocarbons which may be produced by the reduction of sapropel in marine sediments may be oxidized by such bacteria *in situ* as rapidly as they are formed, thereby preventing their accumulation in detectable quantities. As a matter of fact, traces of complex compounds such as polyene pigments and their allies are about the only hydrocarbons found in recent sediments with the exception of methane. The abundance of hydrocarbon-oxidizing bacteria in storage tank water suggests that they may play a role in the decomposition of petroleum products. Marine bacteria which oxidize natural and synthetic rubber, $(C_5H_8)_x$, have been described elsewhere by the authors.[5]

It is still indeterminate whether hydrocarbons are utilized in an anærobic environment although there is some evidence that certain substances such as nitrate and possibly sulfate may serve as hydrogen-acceptors in the absence of free oxygen. Low concentrations of hydrocarbons appear to be oxidized quantitatively to carbon dioxide and water, but with larger concentrations, methane and organic acids have been detected as end-products. Adsorbing the hydrocarbons on sand has proved to be a satisfactory method of providing for the dispersion of the hydrocarbons which are virtually insoluble in water thereby rendering them more susceptible to bacterial attack.

[5] ZoBell, C. E., and Grant, C. W., *Science*, 1942.

13929

Amino Acid Fermentations by Anaerobic Bacteria.

BARTLEY P. CARDON. (Introduced by K. F. Meyer.)
From the Division of Plant Nutrition, University of California, Berkeley, Calif.

Although many anærobic bacteria decompose proteins and the products of protein hydrolysis, only a few species are known to be able to satisfy their energy requirements by the fermentation of single amino acids or other nitrogenous compounds. These species are *Clostridium tetanomorphum* and *Cl. cochlearium*[1,2] which ferment glutamic acid and *Cl. acidi-urici* and *Cl. cylindrosporum*[3,4] which ferment uric acid and some other purines. Possibly *Cl. botulinum*,[5] *Cl. sporogenes*,[6] and *Cl. tetani*[7] should also be in-

[1] Barker, H. A., *Enzymologia*, 1937, **2**, 175.

[2] Barker, H. A., *Arch. f. Mikrobiol.*, 1939, **10**, 376.

[3] Barker, H. A., and Beck, J. V., *J. Bact.*, 1942, **43**, 291.

[4] Barker, H. A., and Beck, J. V., *J. Biol. Chem.*, 1941, **141**, 3.

[5] Clifton, C. E., *J. Bact.*, 1940, **39**, 485.

[6] Hoogerheide, J. C., and Kocholaty, W., *Biochem. J.*, 1938, **32**, 949.

[7] Clifton, C. E., *J. Bact.*, 1942, **44**, 179.

cluded in this group since cell suspensions of all 3 species can decompose single amino acids anærobically. However, it is not yet certain that these decomposition reactions can satisfy the energy requirements for growth of these bacteria.

Recent work in this laboratory has resulted in the discovery of 2 additional obligately anærobic bacteria that derive energy by the fermentation of single amino acids. Both organisms undoubtedly represent new species.

The first organism is a spindle-shaped, spore-forming, motile, Gram-negative rod which ferments alanine, serine and threonine as well as lactate and pyruvate. Methionine, valine, cysteine and aspartate are also attacked but much more slowly. Other amino acids and carbohydrates are not fermented.

The fermentation products are very similar to those produced by the genus *Propionibacterium* since all C_3 substrates give rise to propionic and acetic acids approximately in accordance with the following equations:

$$3\ CH_3CHNH_2COOH + 2\ H_2O \rightarrow 3\ NH_3 + 2\ CH_3CH_2COOH + 1\ CH_3COOH + 1\ CO_2$$

$$3\ CH_2OHCHNH_2COOH + 1\ H_2O \rightarrow 3\ NH_3 + 1\ CH_3CH_2COOH + 2\ CH_3COOH + 2\ CO_2$$

$$3\ CH_3CHOHCOOH \rightarrow 2\ CH_3CH_2COOH + 1\ CH_3COOH + 1\ CO_2 + H_2O$$

$$3\ CH_3COCOOH + 1\ H_2O \rightarrow 1\ CH_3CH_2COOH + 2\ CH_3COOH + 2\ CO_2$$

Threonine, however, gives butyric instead of propionic acid as the characteristic product:

$$3\ CH_3CHOHCHNH_2COOH + H_2O \rightarrow 3\ NH_3 + 2\ CH_3CH_2CH_2COOH + 1\ CH_3COOH + 2\ CO_2$$

The second organism is a Gram-negative coccus that ferments glycine readily. Very few other amino acids are attacked and none is decomposed as rapidly as glycine. The organism may therefore prove useful for the identification and estimation of this amino acid. The fermentation of glycine proceeds approximately in accordance with the equation:

$$4\ CH_2NH_2COOH + 2\ H_2O \rightarrow 4\ NH_3 + 3\ CH_3COOH + 2\ CO_2$$

Under certain conditions, a considerable amount of hydrogen (as much as 0.35 moles per mole of glycine fermented) is also produced, while the carbon dioxide is increased and the acetic acid decreased.

Since the above reactions are carried out by growing cultures as well as by cell suspensions there can be no doubt that they provide the main energy source for these bacteria.

13930 P

Preparation and Partial Analysis of a Pancreatic Protein Material of Unusual Growth-Promoting Properties.

LOUIS A. KAZAL, ROBERT J. WESTFALL, LEON S. CIERESZKO, EDWIN A. RISLEY AND L. EARLE ARNOW.

From the Laboratory of Biochemistry, Medical-Research Division, Sharp and Dohme, Inc., Glenolden, Pennsylvania.

During the course of an investigation of the chemistry of beef pancreas, two protein materials were prepared. The experiments described in the following paper[1] indicate that one of these (P-1) has unusual growth-promoting properties as tested with white rats.

Preparation. Frozen beef pancreas was finely minced and treated for 24 hr with acid 90% 3A alcohol (30 gallons of alcohol and 1 liter of concentrated hydrochloric acid for each 100 lb of pancreas). The insoluble residue was centrifuged off and thoroughly washed with 70% 3A alcohol (28 gallons of alcohol for the residue from 100 lb of glands). After separation with the centrifuge, the air-dried insoluble material was

1 White, A., and Sayers, M. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 270

treated continuously with warm acetone and the treatment continued until nearly all of the lipid had been removed. Acetone was removed by air-drying or by the use of the vacuum oven. P-1a and P-1b were prepared from 2 separate lots of this acetone-treated material by successive passages through a meat grinder and a sieve (20 mesh). P-1c was passed through the meat grinder, but was not sieved. P-1d was finely powdered in a hammer mill.

TABLE I.
Partial Analysis of Preparations.

	Analyses of P-1a, P-1b, P-1c and P-1d		Analysis of P-2
	Average, %	Range, %	%
Moisture	9.2	6.6 -10.8	13.9
Ash	2.2	1.6 - 2.6	5.35
Lipid	1.9	0.05- 3.2	1.0
Nitrogen*	16.2	15.8 -16.5	16.7
Phosphorus*	1.56	1.48- 1.62	1.51
Tryptophane*	1.56	1.47- 1.62	1.86
Tyrosine*	3.85	3.64- 4.06	4.77

*Moisture-free, ash-free, lipid-free basis.

The alcoholic centrifugate and filtrate mentioned above were combined and adjusted to pH 8 with ammonium hydroxide. The insoluble material that separated was removed by filtration, thoroughly treated with hot acetone, and air-dried. This material was designated P-2. It contained some P-1 material that was not removed when the alcoholic mixture was centrifuged.

Analysis. Analytical figures for the various preparations are given in Table I. Tyrosine and tryptophane analyses were carried out by the method of Lugg,[2] without the use of stannite. Phosphorus was determined by the method of Benedict and Theis,[3] or by the method of Fiske and Subbarow.[4] Values found by the two were in close agreement. The Klett-Summerson photoelectric instrument was used for the colorimetric measurements. The nitrogen values were obtained by micro-Kjeldahl analysis.

P-1d was analyzed for prine nitrogen by the method of Graff and Maculla.[5] The value found was 1.31% (8.1% of the total nitrogen). The trichloracetic acid-soluble inorganic phosphorus content of this same sample was found to be 0.146% (9.9% of the total phosphorus). The purine nitrogen and phosphorus figures suggest a nucleic acid content in the neighborhood of 10-14% of the dry, lipid-free, ash-free protein. The atomic purine nitrogen:phosphorus ratio found for this sample (2.18) is fairly close to the ratios calculated from the data of Loring[6] for the nucleic acid of tobacco mosaic virus (1.96-2.16).

We wish to express to Mr. H. J. Rock, of the Glandular Products Department, Sharp and Dohme, Inc., our thanks for his very helpful coöperation.

2 Lugg, J. W. H., *Biochem. J.*, 1937, **31**, 1422.

3 Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, 1924, **61**, 63.

4 Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

5 Graff, S., and Maculla, A., *J. Biol. Chem.*, 1935, **110**, 71.

6 Loring, H. S., *J. Biol. Chem.*, 1939, **130**, 251.

13931 P

Accelerated Rat Growth Rate on Dietary Nitrogen Obtained from Pancreas.*

Abraham White and Marion A. Sayers.

From the Department of Physiological Chemistry, Yale University School of Medicine, New Haven.

During the course of an investigation of the problem of the parenteral utilization of nitrogen derived from protein sources, it became of importance to obtain a readily-available, nutritively-adequate protein as starting material for these studies. One of the methods employed for establishing the nutritive value of the various proteins has been a study of the comparative rates of rat growth on diets which differed from one another only in the kind of dietary protein nitrogen. The standard level of protein used has been 18% of the basal diet, and for each protein source the protein content was calculated on the basis of percent nitrogen of the preparation multiplied by the factor 6.25. Each source of protein nitrogen was thus incorporated in the basal diet in comparable quantities, and, although this nitrogen was not, in all instances, entirely in the form of protein, the various diets used were approximately equivalent in their protein content.

The preliminary data which have been obtained are striking in that they reveal a marked rate of rat growth on a basal diet in which the nitrogen is furnished by a protein preparation derived from pancreas tissue. Indeed, in terms of rate of rat growth, this pancreas product is definitely superior to casein insofar as its capacity to promote the growth of the rat is concerned.

Experimental. Male, white rats of the Yale strain were placed at weaning on one of the several basal diets shown in Table I. In addition, each animal received a daily supplement of 400 mg of dried yeast† and 200 mg of cod liver oil. The basal diets were fed *ad libitum*. The rats were weighed twice weekly; an accurate measure of food consumption was kept.

The pancreas materials used were kindly furnished by The Laboratory of Biochemistry, Medical-Research Division, Sharp & Dohme, Inc.; the preparation and preliminary characterization of these products will be found in the preceding communication.[1] This same laboratory also supplied the soy bean protein and the beef serum preparations.‡ The heat-coagulated beef serum protein was obtained as follows: Whole dried serum was heated to boiling in water solution at pH 5.0. After centrifugation, the gummy, lipid-containing material that floated to the top was skimmed off and discarded. The coagulated protein was washed with water and dried with acetone. The soy bean protein was prepared essentially as described by Smith and Circle.[2] Commercial "soy bean protein" was extracted with 0.05 N NaOH (16 liters per kg of protein). The extract (pH 7.8) was filtered and adjusted to pH 4.1-4.2 with sulfuric acid and the precipitate ("glycinin") filtered, washed with water and dried with acetone. The dried protein was placed in the oven at 105°C for 100 min. This "toasting" procedure was used because of the report[3] that "toasted" soy bean protein has a higher nutritive value than does unheated material.

* This investigation was aided by a grant from the Fluid Research Fund, Yale University School of Medicine, and by a grant to Dr. C. N. H. Long from the Josiah Macy, Jr., Foundation.

† Northwestern Yeast Co., Chicago, Illinois.

1 Kazal, L. A., Westfall, R. J., Ciereszko, L. S., Risley, E. A., and Arnow, L. E., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 268.

‡ The whole dried beef serum was made available to Sharp & Dohme through the courtesy of Dr. David Klein of the Wilson Laboratories.

2 Smith, A. K., and Circle, S. J., *Ind. Eng. Chem.*, 1939, **31**, 1284.

3 Wilgus, H. S., Jr., Norris, L. C., and Heuser, G. F., *Ind. Eng. Chem.*, 1936, **28**, 586.

TABLE I.

Percentage Composition of Basal Diets Employed in Growth Studies.

Diet	A	B	C	D	E	F	G
Sucrose	15	15	15	15	15	15	15
Salts (Osborne and Mendel*)	4	4	4	4	4	4	4
Crisco	25	25	25	25	25	25	25
Starch	34.6	38	34	34	30	31	35
Commercial Casein	21.4						
Pumpkin seed globulin		18					
Pancreas protein (P-1)			22				
Pancreas protein (P-2)				22			
Dried whole beef serum					26		
Heat-coagulated beef serum protein						25	
Soy bean protein							21

*Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, **37**, 557.

TABLE II.

Average Daily Weight Gains and Food Consumptions of Rats on Various Basal Diets.

Diet	No. of animals	Avg daily wt gain, g	Avg daily food consumption, g
A (commercial casein)	3	4.4	8.0
B (pumpkin seed globulin)	5	2.3	5.7
C (pancreas protein; P-1*)	14	5.9	10.8
D (pancreas protein; P-2)	4	3.0	7.0
E (dried whole beef serum)	4	2.5	5.7
F (heat-coagulated beef serum protein)	3	1.9	4.7
G (soy bean protein)	4	1.0	5.5

*The various P-1 preparations mentioned in the preceding paper[1] appear to be equally effective in maintaining an accelerated growth rate.

The casein was a commercial product.§ Pumpkin seed globulin was prepared from *Cucurbita moschata*.[4]

In Table II are summarized the data obtained for growth and food consumptions of the animals during the first 28 days on the diets after weaning. It is apparent that young rats ingesting a basal diet in which the so-called whole pancreas residue product (P-1) supplies the protein nitrogen (except for that derived from the daily yeast supplement) exhibit a marked rate of growth. This rate is considerably better than that observed for animals on basal diets containing a variety of other kinds of protein. The food consumption of rats ingesting the basal diet containing the pancreas preparation is also markedly higher than that of animals eating any one of the other basal diets.

It may be added that, although the data in Table II represent only the growth rate for a 28-day period, the rats on the pancreas and casein basal diets have been permitted to continue on these rations. In these experiments, the daily vitamin supplement has been doubled for each animal as it reached a body weight of 125 g. The animals ingesting the whole pancreas protein residue-containing basal diet continue to exhibit the same high rate of body weight gain until a body weight of approximately 200 to 250 g has been attained. After this time, the rate of weight gain becomes less but continues to be definitely greater than that of litter-mate animals of the same age on a casein-containing basal diet. For example, one of the animals on the diet containing the whole pancreas protein residue has at this writing attained a body weight of 640 g at an age of 145 days, having shown a total weight gain of 600 g since weaning; casein-ingesting, litter-mate controls of the same age now weigh, on the average, 490 g. The ratio of body length to body weight of all animals is quite similar.

§ Lister Bros., New York.

[4] Vickery, H. B., Smith, E. L., Hubbell, R. B., and Nolan, L. S., *J. Biol. Chem.*, 1941, **140**, 631.

13932

Effect of Oxidation-Reduction on Germicidal Efficiency of Some Metallic Salts.

HOWARD L. GUEST AND A. J. SALLE.

From the Department of Bacteriology, University of California, Los Angeles.

It is well known that the inorganic ferrous and ferric salts, when tested individually, exhibit very little or no germicidal activity against *Eberthella typhosa* and *Staphylococcus aureus*. However, if an oxidized and a reduced salt, such as ferric and ferrous chlorides, are mixed to form an oxidation-reduction system a pronounced germicidal action occurs.

The salt pairs cannot be combined haphazardly but must be mixed in certain definite proportions for maximum germicidal activity to occur. In the case of the chlorides the most effective germicidal action occurred when the salts were mixed in the proportion of 2 moles of ferric chloride and one mole of ferrous chloride. Using a mixture of the 2 salts in this proportion a dilution of 1:100 was germicidal against *Staphylococcus aureus* in 10 min at 37°C.

The sulfates of the 2 iron salts behaved in a similar manner, indicating that the positive radical is the effective portion of the molecule. In this case the most effective combination was in the proportion of one mole of each salt. The results are given in Table I.

TABLE I.

Effectiveness of Ferrous and Ferric Salts Used Singly and in Combinations Against *Staphylococcus aureus.*

Salt	Killing dilution in 10 min at 37°C*
$FeCl_2$	1:10 failed to kill
$FeCl_3$	1:25
$FeCl_2 + 2FeCl_3$	1:100
$FeSO_4$	1:10 failed to kill
$Fe_2(SO_4)_3$	1:30
$FeSO_4 + Fe_2(SO_4)_3$	1:75

*Dilutions of the combination of chlorides were prepared by mixing 1 g mole of $FeCl_2$ (198.8 g) and 2 g moles of $FeCl_3$ (540.6 g). A 1:100 dilution means 1 g of the mixture diluted to 100 cc with distilled water.

The most effective combination of the sulfates was 1 g mole of each salt.

Continuing with this same line of reasoning it was found that salts of tin and manganese used singly and in combination gave results similar to the iron compounds. The results of tests on the tin salts are given in Table II. It may be seen that stannous and stannic chlorides tested individually exhibited only a mild germicidal action whereas a more pronounced killing effect occurred when the two salts were mixed in proper proportion.

The germicidal effect is not limited to salts having a common metallic ion. Salts of different metallic cations and anions may be crossed with the same general effect. The important point is to have an oxidation-

TABLE II.

Effectiveness of Stannous and Stannic Chlorides Used Singly and in Combination Against *Staphylococcus aureus.*

Salt	Killing dilution in 10 min at 37°C*
$SnCl_2$	1:40
$SnCl_4$	1:50
$SnCl_2 + SnCl_4$	1:140

*The most effective combination of the salts was 1 g mole of each.

TABLE III.

Effectiveness of Various Metal Salts Tested Singly and in Combinations Against *Staphylococcus aureus.*

Salt	Killing dilution in 10 min at 37°C*
$FeCl_2 + SnCl_4$	1:130
$FeCl_3 + SnCl_2$	1:120
$AgNO_3$	1:100
$AgNO_3 + 2Fe(NO_3)_3 + FeSO_4$	1:800 of silver nitrate
$HgCl_2$	1:16,000
$HgCl_2 + FeSO_4$	1:40,000 of mercuric chloride
$MnSO_4$	1:10 failed to kill
$MnSO_4 + SnCl_4$	1:120
$MnSO_4 + Fe_2(SO_4)_3$	1:100

*The salts in the various combinations were mixed in the proportions given in the table. Unless otherwise noted the dilutions refer to the mixtures.

reduction system for increased germicidal efficiency to occur. The results of tests on a number of metallic salts used singly and in combinations against *Staphylococcus aureus* are given in Table III. It may be seen that in every case a mixture of two salts, one in a higher state of oxidation than the other, resulted in an increased germicidal action over that of the constituent salts tested singly.

The increased killing power of the combinations is essentially a function of the positive metallic ions. A mixture of sodium sulfite and sodium sulfate exhibited no increased action over that of the same salts tested singly.

The results indicate that the phenomenon is a general one. There was not a single exception to the rule that 2 salts mixed to produce an oxidation-reduction system increased markedly the germicidal efficiency of the same salts when tested individually.

Summary. Inorganic metallic salts, which are not germicidal or only slightly so, may exhibit a pronounced killing effect when mixed in certain combinations and proportions. In order that increased germicidal action may occur the salts must be mixed to produce an oxidation-reduction system. The phenomenon is a function of the positive metallic ions; the negative ions apparently play no part in the reaction.

In a later paper it will be shown that the phenomenon is not limited to inorganic compounds only but may be applied to mixtures of inorganic salts and organic compounds.

13933 P

A Rapid Test for the Activity of Certain Antibiotic Substances.

GEOFFREY RAKE, CLARA M. MCKEE AND HELEN JONES.

From The Squibb Institute for Medical Research, New Brunswick, N.J.

The need for a rapid assay of activity of antibiotic substances was emphasized during investigations on such substances. Such an assay of the substrate of mold growth or of chemical fractions would greatly facilitate work.

In working with aspergillic acid, active crystalline material isolated from a strain of *Aspergillus flavus*,[1,2] an antibacterial serial dilution test with *Streptococcus pyogenes* was used. To replace this test, it was thought that aspergillic acid might interfere rapidly with the luminescence produced by luminescent bacteria to a degree which could be directly correlated with the antibacterial activity. Accordingly, a culture of *Photobacterium fischeri* was obtained from the National Type Collection. This culture grew well at room temperature in ocean water with 0.2% peptone or on artificial sea water,* viz.:

NaCl	26.7 g
KCl	0.71 g
$CaCl_2 \cdot 2H_2O$	1.52 g
$MgCl_2 \cdot 6H_2O$	5.11 g
$MgSO_4 \cdot 7H_2O$	6.81 g
Distilled water	1000 ml

again with 0.2% peptone. On such fluid media faint luminescence was visible in 6 hr and maximal luminescence in about 24.

It was soon found that this antiluminescent test was applicable for the assay of aspergillic acid, but not for penicillin. The work here reported deals largely with aspergillic acid.

For the test 0.5 ml of sea water media was placed in a series of 13 x 100 mm tubes. To the first was added 0.5 ml of the solution to be tested. The contents of this tube were mixed and 0.5 ml transferred to the next, the process being continued to give 2-fold dilutions. To each tube was now added 0.5 ml of an undiluted 24-hr culture of

1 White, E. C., *Science*, 1940, **92**, 127.

2 White, E. C., and Hill, J. H., *J. Bact.*, in press.

* We express our gratitude to Dr. W. H. Cole of Rutgers University for furnishing this formula.

Photobacterium fischeri and the mixtures shaken. The tubes were again shaken just before the test was read to oxygenate and produce maximal luminescence. Readings were made in the dark-room with the dark-adapted eye and the degree of luminescence recorded as from 1+ to 4+. A solution of purified aspergillic acid with 256 units per mg was always included and the results of all unknowns were read in comparison with this standard. Tests have proven clear cut, and, except in certain early filtrates, there was at most only one tube between full luminescence and complete blacking out.

TABLE I.

Comparison of Antiluminescent and Antibacterial Activities of Various Antibiotic Substances.

Substance	Smallest amount in γ showing activity	
	Antiluminescent test	Antibacterial test using 1,500,000 *Streptococcus pyogenes*—Strain C203
Aspergillic Acid	15	2
Gliotoxin	18	2
Clavacin*	12	63
Actinomycin A*	47	0.7
Penicillin†	1650	0.06
Gramicidin‡	> 500	0.002
Gramidinic Acid‡	> 500	0.23
AP 21‡	> 500	0.31

*Supplied through the courtesy of Dr. S. A. Waksman of the N.J. Agricultural Experiment Station, Rutgers University.

†This preparation of penicillin was the purest tested and had 350 Florey units per mg.

‡Supplied through the courtesy of Dr. J. C. Hoogerheide of the Biological Laboratories of E. R. Squibb and Sons. AP 21 is the active material obtained from a spore forming soil anærobe.

Readings made after the mixtures had stood for 30 min at 25°C were the most significant. At shorter intervals interference with luminescence was still proceeding.

Comparison of the two tests showed that in 100 consecutive tests on crystalline aspergillic acid or on crude mold substrates, 33 gave identical titers, while 33 were lower with the antiluminescent test and 34 higher. Of the latter 67 tests only 9 showed a variation as great as 2-fold and none showed greater, a degree of variation encountered in a series of antibacterial tests run in duplicate.

Evidence that the antiluminescent test can replace the antibacterial, is shown by the fact that results obtained on the basis of the first test have been in practice just as satisfactory as those based on the second. In some filtrates from old cultures of the mold on brown sugar-tryptone medium the results obtained in the antiluminescent test differ greatly from those obtained in the antibacterial. Such results indicate the presence of unknown active substances other than aspergillic acid.

An attempt has been made to apply this rapid test for activity to the assaying of other antibiotic substances (Table I). Gliotoxin behaves like aspergillic acid. Clavacin from *(Aspergillus clavatus)* and actinomycin A (from *Actinomyces antibioticus*) show somewhat similar correlation. Products from certain soil bacteria show little if any antiluminescent activity. The same is true of penicillin which substance, although derived from a mold, is very different in its properties from aspergillic acid, clavacin, or gliotoxin. Tests have indicated that crude filtrates from *Penicillium notatum* contain some factor other than penicillin which has some power to destroy luminescence.

Summary. A rapid method for the assay of aspergillic acid in crude or pure state has been described. This method, based on antiluminescent activity, may have wider application in the investigation of many antibiotic substances derived from molds and actinomyces.

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Activity of Penicillin Against Strains of Pneumococci Resistant to Sulfonamide Drugs.

CLARA M. MCKEE AND GEOFFREY RAKE.

From the Division of Microbiology, The Squibb Institute for Medical Research, New Brunswick, N.J.

We[1] reported work on penicillin in which brief mention was made of protection with penicillin against strains of pneumococci resistant to the sulfonamides *in vivo*. The work has been expanded and the present paper gives a fuller report on the subject.* At about the same time Powell and Jamieson[2] published a report on protection with penicillin and sulfapyridine against 3 strains of pneumococci Types I, II and III and their derived "sulfapyridine fast" strains. All strains except the Type I parent strain were highly resistant to sulfapyridine and all were susceptible to the action of penicillin. In the case of the Type I strain the acquisition of resistance to sulfapyridine did not change its susceptibility to penicillin.

In the work reported here 4 strains naturally resistant to the sulfonamides were used. Two Type II strains No. 617 and No. 807 were completely resistant to sulfadiazine when it was injected intraperitoneally or subcutaneously in mice or when it was administered in the food in either 0.25 or 0.5%. That is, all treated animals died, although the length of life was slightly longer in the treated than in the untreated groups. Two other strains, Type I P and Type VIII 755, were resistant but to a lesser degree than the two Type II strains since some of the treated mice survived and the difference between the survival time of treated and control mice was greater. Table I shows the relative resistance *in vivo* of these 4 strains and 2 susceptible strains. *In vitro* susceptibility of the same strains to sulfadiazine and penicillin is also shown.

There was no correlation between the resistance of the cultures to sulfadiazine *in vivo* and *in vitro*. The two strains Type II 617 and 807, most resistant *in vivo*, were more susceptible *in vitro* than the strain Type I 710 which was susceptible *in vivo*. A direct relationship seemed to exist between virulence of cultures and *in vivo* resistance to sulfadiazine. Therefore, these strains cannot be regarded as sulfonamide fast in the usually accepted sense and will be termed sulfonamide resistant. In later experiments it was shown that rapid serial passage through mice, either normal or sulfadiazine treated, rendered PnI P and PnVIII 755 more resistant to sulfadiazine apparently merely by increasing the virulence.

It was found that all strains were susceptible to the action of penicillin. In consequence more extensive experiments were carried out to determine the degree of protection achieved with penicillin as compared with sulfadiazine.

Swiss mice weighing approximately 20 g were used as the test animals. The mice were infected intraperitoneally with 0.5 cc of a 2 x 10^{-7} dilution of a 16-hr blood broth culture. This dose contained 50 to 60 organisms and, since all cultures were highly virulent for mice, represented at least 10 lethal doses. All stock cultures when not in use were kept in the frozen state. The resistant strains were passed through mice just before use and it was found that the recovered cultures killed all mice in a 10^{-7} dilution. The cultures were grown in a beef heart infusion broth containing rabbit defibrinated blood. In the course of any series of experiments it has been customary to use a culture recovered from a control mouse from the previous experiment. The penicillin-treated

[1] McKee, C. M., and Rake, G., *J. Bact.*, 1942, **43**, 645.

* The penicillin used in the present studies was supplied through the kindness of Dr. J. C. Hoogerheide of the Biological Laboratories of E. R. Squibb and Sons.

[2] Powell, H. M., and Jamieson, W. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 387.

TABLE I.

Resistance of Pneumococcus Cultures to 0.5% Sulfadiazine *in vivo* and Susceptibility to Sulfadiazine and Penicillin *in vitro*.

	In vivo				*In vitro*	
		No. of mice				
Culture	Sulfadiazine	L	D	Avg length* of life	Sulfadiazine†	Penicillin†
PnII 617	0.5% in food	0	20	37 hr	4	0.3
PnII 807	"	0	20	58 "	4	0.3
PnI P	"	5	7	5 da	15.6	0.6
PnVIII 755	"	8	14	8 "	15.6	0.15
PnI 710	"	10	0		31	0.6
PnIII S	"	10	0		2	0.6

*The average length of life of control mice for the 2 Type II strains was 22 hours; for the Type I P and Type VIII strains, 24 hours; and for the 2 susceptible strains, Type I 710 and Type III S, 44 hours.

†The least amount in γ per cc which inhibited growth of approximately 800 organisms.

TABLE II.

Representative Protocols Showing Comparative Protection with Penicillin and Sulfadiazine.

Pneumococcus strain	Treatment	Doses	Florey units	No. of mice Lived	No. of mice Died	Avg length of life in hr
PnII 617	Penicillin i.p.	1	10	18	2	
		1	1	4	2	
		6	10	9	1	
		6	0.2	9	11	
	Sulfadiazine 0.5% in food			0	10	< 31
	Control			0	10	< 22
PnII 807	Penicillin s.c.	1	100	10	0	
		1	10	0	5	
		6	10	3	2	
	Sulfadiazine 0.25% in food			0	10	< 48
	Control			0	10	< 24
PnI P	Penicillin i.p.	1	10	20	0	
		1	1	19	1	
	Penicillin s.c.	1	100	20	0	
		1	10	4	16	
	Sulfadiazine 0.25% in food			0	10	< 48
	" 0.5% " "			0	10	< 92.6
	Control			0	10	< 24
PnVIII 755	Penicillin i.p.	1	100	10	0	
		1	10	10	0	
		1	1	9	1	
		6	10	10	1	
		6	1	10	1	
	Penicillin s.c.	1	100	10	0	
	Sulfadiazine 0.25% in food			0	10	< 54.4
	" 0.5% " "			0	10	<113.8
	Control			0	10	< 25.6

mice were injected either intraperitoneally or subcutaneously immediately after infection. In some cases only one injection was given, at other times three injections a day for two days were made. Injections were spaced about 4 hr apart at 9 A.M., 1 and 5 P.M. No injections were made during the night. A sodium salt of penicillin was used, the potency of which was 100 Florey units per mg. When administered intraperitoneally as little as one Florey unit or 0.01 mg protected a large number of the mice so treated. When treatment was given subcutaneously, *i.e.* at a distance from the site of infection, more penicillin was necessary for protection. Ten Florey units gave some protection and 100 Florey units complete protection. Sulfadiazine was administered in the food in either

TABLE III.

Summary of Comparative Protection with Penicillin and Sulfadiazine.

Pneumococcus strain	Dilution of culture	Treatment				No. of mice	
		Material	Route	Dose	Florey units	Lived	Died
PnII 617	10-7	P*	i.p.	1	10	24	2
	,,	,,	,,	1	1	13	3
	,,	,,	,,	6	10	39	2
	,,	,,	,,	6	1	6	0
	,,	,,	,,	6	0.2	15	11
	10-6	,,	,,	1	1	3	7
	10-5	,,	,,	1	100	5	5
	,,	,,	,,	6	10	10	3
	10-4	,,	,,	6	10	3	3
	10-7	,,	s.c.	1	100	16	0
	,,	,,	,,	1	10	1	4
	,,	,,	,,	6	10	1	4
	10-5	,,	,,	1	100	1	9
	,,	,,	,,	1	50	0	10
	10-7	SD*		.25%		0	15
	,,	,,		.5%		0	46
	,,	C*				0	64
PnII 807	10-7	P	i.p.	1	10	22	3
	,,	,,	,,	1	1	18	7
	,,	,,	,,	6	10	32	0
	,,	,,	,,	6	1	11	4
	,,	,,	,,	6	0.2	1	4
	10-6	,,	,,	1	100	10	1
	,,	,,	,,	6	10	5	1
	10-5	,,	,,	6	10	0	10
	10-7	,,	s.c.	1	100	16	0
	,,	,,	,,	1	10	0	5
	,,	,,	,,	6	10	3	2
	10-6	,,	,,	1	100	9	1
	,,	,,	,,	1	50	9	1
	10-7	SD		.25%		0	15
	,,	,,		.5%		0	46
	,,	C				0	64
PnIP	10-7	P	i.p.	1	10	51	1
	,,	,,	,,	1	1	46	6
	10-6	,,	,,	6	10	20	0
	10-7	,,	s.c.	1	100	52	0
	,,	,,	,,	1	10	21	31
	,,	SD		.25%		0	20
	,,	,,		.5%		0	32
	,,	C				1	31
PnVIII 755	10-7	P	i.p.	1	10	20	0
	,,	,,	,,	1	1	19	1
	,,	,,	,,	6	10	20	0
	,,	,,	,,	6	1	20	0
	10-6	,,	,,	6	10	20	0
	10-7	,,	s.c.	1	100	20	0
	,,	SD		.25%		0	20
	,,	,,		.5%		0	20
	,,	C				0	20

* P = Penicillin, 100 Florey units per mg.
SD = Sulfadiazine in food.
C = Control.

0.25 or 0.5%. All mice were put on the drug-containing diet 2 days before infection. Those mice receiving 0.5% drug in the food lived a little longer than those receiving 0.25%. Typical protocols of experiments with the 4 strains of pneumococci are given in Table II. In all cases the infecting dose was .5 cc of a 2 x 10^{-7} dilution given intraperitoneally. Since penicillin is so quickly excreted, repeated treatments at short intervals are usually given. It is of interest, therefore, as shown in the above protocols, that we obtained excellent protection with a single dose. In the case of PnII 617 it will be seen that a single dose of 10 Florey units gave as good protection as repeated doses. This suggests that penicillin exerts bactericidal as well as bacteriostatic action.

Pooled results of all experiments with PnII 617, and PnII 807 and of those experiments with PnI P and PnVIII 755 made after the strains had been rendered more virulent by mouse passage, are given in Table III. These results include infection with larger numbers of organisms, and in such cases differences in susceptibility of strains to penicillin become apparent. Penicillin afforded some protection against infection with PnII 617 even when the dose of organisms was increased 1000 fold but with PnII 807 it protected only against a ten-fold increase, not against 100 fold. Complete protection against PnI P and PnVIII 755 was achieved with 10 times the usual dose of organisms, the largest number tried.

As mentioned above, when an attempt was made to increase the resistance to sulfadiazine of PnI P and PnVIII 755 it was found that rapid serial passage through either normal or sulfadiazine-treated mice so increased the virulence that all mice treated with sulfadiazine died. Both strains were equally susceptible to the action of penicillin and equally virulent for the control mice. This suggests the possibility that in some other reported cases apparent drug fixation acquired by animal passage may be due, at least in part, to increased virulence.

An effort was made to produce penicillin resistant strains. Cultures of PnII 617 and 807 recovered from penicillin treated mice were grown for 8 transfers in broth containing the greatest amount of penicillin which would permit growth. When tested *in vitro* the penicillin passage strains proved to be twice as resistant as the unpassed strains to the action of penicillin. However, when tested in mice no difference in resistance was observed. Abraham, Chain *et al.*[3] were able to adapt a *Staphylococcus aureus* culture to penicillin by growing the culture in penicillin-containing broth so that after a few daily subcultures the organism grew in twice the previously inhibitory concentration. In 9 weeks, with subcultures every few days an adaptation to a 30-fold concentration was reached and in a further 7 weeks the adapted strain grew in a concentration 1000 times greater than that which inhibited the original culture. No *in vivo* tests were made with the penicillin resistant strain. Passage with penicillin either *in vitro* or *in vivo* over a longer period of time might render our pneumococcus cultures more resistant.

Summary. Four strains of pneumococci naturally resistant to sulfonamide action have been shown by mouse protection tests to be readily susceptible to the action of penicillin. When infection and treatment were both given intraperitoneally a single dose of one Florey unit protected 84%, and 10 Florey units protected 95% of the treated mice; 6 doses of one Florey unit per dose protected 90% and 6 doses of 10 Florey units protected 99% of the treated mice. When infection was given intraperitoneally and treatment subcutaneously a single dose of 10 Florey units protected 35%, and 100 Florey units protected 100% of the treated mice.

Cultures of PnII 617 and 807 after growth in penicillin broth for a short time were resistant to twice the previously inhibitory concentration. No difference in resistance could be shown by mouse protection test.

[3] Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **2**, 177.

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Haemobartonella tyzzeri in Colombia.

HERNANDO GROOT. (Introduced by David Weinman.)
From the Hygienic Laboratory of Nariño (Colombia).

This paper represents the results of our studies on splenectomized guinea pigs from Colombia, in relation to bartonella infection.

Weinman and Pinkerton[1] described a new species of bartonella from the guinea pig. It was called *Bartonella tyzzeri.* Later Tyzzer and Weinman,[2] on the basis of striking differences between the human and animal bartonellas, proposed a division of the genus Bartonella into Bartonella *sensu strictu* and Hæmobartonella, the latter to include all animal bartonellæ known at the time. The guinea pig parasite thus becomes *H. tyzzeri.* The organism was found in Peru in 4 of 23 splenectomized guinea pigs. Infected red cells appeared 5 to 9 days after operation and remained visible for a period varying between a few days and a month. *H. tyzzeri* appears as rods 1.4 to 4 μ long and 0.25 μ wide. The surface of the rods sometimes shows granular swellings. The poles are frequently enlarged. Short rods, averaging 0.8 by 0.2 to 0.3 μ, may also occur. Round, coccus-like forms may be observed. Sometimes the parasite takes a bipolar stain.

Of 20 locally obtained Colombian guinea pigs which we had occasion to splenectomize, one was naturally infected with a bartonella morphologically identical with *H. tyzzeri,* Weinman and Pinkerton. In this infected animal, the parasites appeared on the 8th day after operation. They continued to appear until the 28th day when the guinea pig died accidentally.

The parasite could be transmitted to 2 out of 3 splenectomized, bartonella-free guinea pigs, after intraperitoneal injection of 0.5 cc of infected blood. In one of these 2 guinea pigs, bartonellas appeared in the peripheral blood 5 days after the injection and were visible during two days; in the other guinea pig the parasites appeared on the 19th day following the injection; 35 days later they were still present in the blood stream.

[1] Weinman, D., and Pinkerton, H., *Ann. Trop. Med. and Parasitol.,* 1938, **32**, 215.

[2] Tyzzer, E. E., and Weinman, D., *Am. J. Hygiene,* 1939, **30**, Sec. B, 141.

13936

In vitro Effect of Tyrothricin and Tyrocidine Hydrochloride on Polymorphonuclear Leucocytes.

MARY PRESTON CLAPP. (Introduced by Stuart Mudd.)
From the Department of Public Health and Preventive Medicine, University of Pennsylvania.

This paper reports the effect of tyrothricin and tyrocidine hydrochloride upon exudative rabbit polymorphonuclear leucocytes and upon their phagocytic action *in vitro.* In high concentrations these substances caused cytoplasmic and nuclear disintegration; in lower concentrations altered staining reactions were observed. When there was no apparent microscopic injury to the cells, phagocytosis of pneumococci occurred as expected. It was also found that the presence of serum afforded some protection for the cells from the effects of these substances.

Dilutions of tyrothricin and tyrocidine hydrochloride were mixed with constant amounts of suspensions of a Type I mucoid strain of pneumococcus and of rabbit leucocytes, which were obtained according to the

TABLE I.

Effect of Tyrothricin and Tyrocidine Hydrochloride on Rabbit Leucocytes.

	Tyrothricin							
Rabbit leucocytes	Undil.	1/40	1/400	1/4000	1/40,000	1/400,000	1/4,000,000	Saline
In absence of serum	+++	++	++	+	0	0	0	0
*With normal rabbit serum	++	+	0	0	0	0	0	0
*With antipneumococcic serum	+++	++	+	+	+	0	0	0

	Tyrocidine hydrochloride					
	1.0 mg	0.1 mg	0.01 mg	0.001 mg	0.0001 mg	Saline
*With antipneumococcic serum	+++	++	+	0	0	0

0—No disintegration.
+—Partial cytoplasmic disintegration.
++—Partial cytoplasmic and nuclear disintegration.
+++—Entire cytoplasmic and nuclear disintegration.
*Serum diluted 1:40; all concentrations are expressed as final concentrations.

TABLE II.

Effect of Tyrothricin and Tyrocidine Hydrochloride on Phagocytosis and Pneumococci.

		Tyrothricin							
	Serum 1/40	Undil.	1/40	1/400	1/4000	1/40,000	1/400,000	1/4,000,000	Saline
Pneumococci Approximately 2,400,000,000 per 0.1 ml	Normal rabbit serum								
	% of phagocytosis	0	0	6	6	6	6	6	6
	Amt of lysis of pneumococci	++	++	+	0	0	0	0	0
	Antipneumococcic serum								
	% of phagocytosis	0	80	80	100	100	100	100	100
	Amt of lysis of pneumococci	++	+	+	0	0	0	0	0

		Tyrocidine hydrochloride					
		1.0 mg	0.1 mg	0.01 mg	0.001 mg	0.0001 mg	Saline
	Antipneumococcic serum						
	% of phagocytosis	0	100	100	100	100	100
	Amt of lysis of pneumococci	0	0	0	0	0	0

0—No lysis.
+—Partial lysis.
++—Complete lysis.
All concentrations are expressed as final concentrations.

method of Mudd and his coworkers.[1] The tyrothricin used was obtained from a culture, "B.G.", received from Dubos and prepared according to directions accompanying the culture.[2] The purified tyrocidine hydrochloride was supplied by Dr. W. F. Verway of Sharp and Dohme, Inc. These mixtures were shaken for 10 min at 37°C in the Boerner-Mudd apparatus[3] and smears were made, which were stained by a special

[1] Mudd, S., Lucke, B., McCutcheon, M., and Strumia, M., *J. Exp. Med.*, 1929, **49**, 779.

[2] Dubos, R. J., personal communication, Dec. 26, 1939.

[3] Boerner, F., and Mudd, S., *Am. J. Med. Sci.*, 1935, **189**, 22.

phagocytic stain.[4] Suitable control experiments were conducted which showed that the media in which the tyrothricin was dissolved had no effect in the dilutions considered.

Table I shows the actions of tyrothricin and tyrocidine hydrochloride on leucocytes in the presence and absence of serum. This finding is typical of the results obtained in 20 instances.

Table II shows the effect of tyrothricin and tyrocidine hydrochloride upon the amount of phagocytosis taking place, recorded as the percentage of leucocytes containing pneumococci when 50 cells were observed, and also upon the pneumococci themselves, expressed by the amount of lysis occurring.

When normal leucocytes in saline suspension are treated with the phagocytic stain employed, the nuclei are stained red and the cytoplasm a purplish blue. It was noted that in some of the lower concentrations of tyrothricin and tyrocidine hydrochloride this staining reaction was reversed, the nuclei appearing blue and the cytoplasm red. The cells exhibiting these peculiar staining reactions were also noted to have ingested more pneumococci than those staining in the normal fashion.

The author wishes to express appreciation for suggestions made by Dr. David B. Lackman during the course of this work.

4 Lucke, B., Strumia, M., Mudd, S., McCutcheon, M., and Mudd, E. B. H., *J. Immunol.*, 1933, **24**, 455.

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The Acute Toxicity of Choline Hydrochloride in Mice and Rats.*

HAROLD C. HODGE AND MAX R. GOLDSTEIN.

From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, N.Y.

Although a number of isolated observations on the acute toxicity of choline have been recorded, no systematic studies are available. The relatively low toxicity of choline is illustrated by the statement of Mott and Halliburton[3] that "We have never succeeded in killing an animal by injection of choline or choline hydrochloride." However, the lethal dose of choline has been estimated for several species as shown in the accompanying text table and the generality has been established that animals either die promptly (within 20 min) from the effects of choline or recover, apparently without permanent injury.

No observations on rats are available.

In the tests reported here, choline hydrochloride (Eastman Kodak Co.) was given in aqueous solutions as indicated (Table I). Death, when it occurred, followed promptly —for large doses within 2-4 min in some of the animals. The symptoms were those previously described by many investigators; in

Toxicity of Choline Hydrochloride.

Species	Lethal dose	Route	Reference
Mice	700 mg/kg	Subcutaneous	1
Cats	35 mg/kg	Intravenous	1,2
Rabbits	1 g/kg	Rectally	5
	0.11 cg/kg	Intravenously	5
	1 g/kg	Subcutaneously	5
Frogs, large	0.1 g	?	4
Frogs, small	0.05 g	?	4

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1 Arai, K., *Arch. ges. Physiol.*, 1922, **193**, 359.

2 Lohmann, A., *Arch. ges. Physiol.*, 1907, **118**, 215.

3 Mott, F. W., and Halliburton, W. D., *Trans. Roy. Soc. Lond.*, B, 1899, **191**, 211.

4 Boehm, R., *Arch. Exp. Path. Pharm.*, 1885, **19**, 87.

5 Dreyfus, L., *C. N. S. B.*, 1920, **83**, 481.

mice, salivation, trembling, jerking, cyanosis, clonic shivering convulsions, respiratory paralysis; in rats, these symptoms with, in addition, a marked bleeding from the eyes which appeared to start from the medial canthus. This hemorrhage was observed in about 60% of the rats dying from the drug.

Mice. One hundred six male and female albino mice weighing 18-26 g were given intraperitoneally various doses of choline hydrochloride as a 2% solution in water. No correlation could be observed between body weight (in these limits) and mortality. The fur around the eyes seemed wet to a distance of about 1 mm; the eyes appeared to darken. On the high doses, all the mice that died did so in 4 min after injection. The L.D. 50 (Table IA) was found to be 320 mg per kg by the method of Bliss.[6]

TABLE I.
Acute Toxicity Data on Choline Hydrochloride for Mice and Rats.

A. Mice—Intraperitoneal Injection of 2% Choline Hydrochloride Solution.

No. Mice	Dose, mg	No. dead	% Mortality
16	5.0	0	0
15	5.6	4	27
15	6.0	7	47
15	6.6	6	40
15	7.0	10	67
15	8.0	13	87
15	10.0	13	87

L.D. 50 (probit kill—5.0) = 6.4 mg per mouse or 320 mg per kg.

B. Rats—Stomach Tube Administration of 67% Choline Hydrochloride Solution.

No. Rats	Dose, g	No. dead	% Mortality
15	.40	0	0
15	.54	3	20
15	.60	3	20
15	.67	6	40
15	.80	9	60
15	1.00	12	80

L.D. 50 (probit kill—5.0) = 0.73 g per rat or 6.7 g per kg.

Rats. Ninety male albino rats of average weight about 150 g but varying from 120-200 g were given by stomach tube various doses of choline hydrochloride as a 67% solution in water. No effect on mortality was observed by this variation in body weight. On autopsy, the blood vessels of the diaphragm and stomach were engorged. The liver and spleen appeared congested. Stomachs were bleached and distended. The hearts stopped in diastole. The L.D. 50 (Table IB) was found to be 6.7 g per kg.

Summary. The analyses of the data indicate an L.D. 50 for choline hydrochloride of the order of 320 mg per kg for intraperitoneal injection in albino mice, and of 6.7 g per kg for stomach tube administration in albino rats.

The authors acknowledge the assistance of Ray Kesel.

[6] Bliss, C. I., *Ann. Appl. Biol.*, 1935, **22**, 134.

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Studies on Antibacterial Action of Sulfonamide Drugs. III. Correlation of Drug Activity with Binding to Plasma Proteins.

BERNARD D. DAVIS* AND W. BARRY WOOD, JR.†

From the Chemical and Biological Division of the Department of Medicine, Johns Hopkins University Medical School, Baltimore, and the Division of Industrial Hygiene, National Institute of Health, Bethesda, Md.

In a preliminary report[1] a correlation was noted between the bacteriostatic power and the protein-binding tendency of 4 sulfonamide drugs: sulfanilamide, sulfapyridine, sulfadiazine, and sulfathiazole. It has been suggested by Woods[2] that sulfonamide bacteriostasis depends upon specific inhibition of an enzymatic reaction involving p-aminobenzoic acid. Further evidence has recently been presented[3-5] supporting this concept. Since all known enzymes are proteins, and since the inhibition of an enzymatic reaction may involve some form of chemical interaction between inhibitor and enzyme, it seemed worthwhile to investigate further the interaction between sulfonamide drugs and proteins. The present paper reports data on the binding to plasma proteins of a larger series of sulfonamide drugs and related compounds. The significance of the data is briefly discussed.

Methods. The method of determining the binding of drug to plasma protein, which is described in detail elsewhere,[6] consisted essentially of determining the concentration of drug in samples of plasma and buffer (pH 7.4) which had been dialyzed against each other until equilibrium was reached. The concentration of drug in the plasma in excess of that found in the buffer was considered to be bound to the protein; the data are reported as the percentage of the value of the unbound drug which is bound per g of protein. All the experiments were performed with a single lot of plasma.

The method of determining the bacteriostatic activity of the various sulfonamide drugs has been described in a previous paper.[4] The minimum concentration of drug, which would prevent visible growth of a controlled inoculum of *E. coli* incubated in a synthetic medium for 5 days at 37°C, was arbitrarily selected as the bacteriostatic end point.

Results. Binding of p-Amino Benzene Sulfonamides. In Table I are presented data on the binding to plasma of 7 homologues of the p-amino series which have in common the p-amino radical but vary in the groups attached to the sulfonamide portion of the molecule. The minimum bacteriostatic concentrations of each drug against *E. coli* are also recorded in Table I. Although the measurement of relative bacteriostatic activity is admittedly crude, there is demonstrated a close correlation between binding tendency and bacteriostatic potency. While this agreement may be coincidental, it suggests that the tendency of a given drug to be bound to plasma protein depends upon properties which likewise are of importance in the mechanism of bacteriostasis.

Binding of Related Compounds. Sulfonamide compounds which do not contain the p-amino group also become bound to protein. It is seen from the data in Table II that ortho- and meta-amino benzene sulfonamide, the less active isomers of sulfanilamide, show as much, and in the case of orthonilamide even more binding than sulfanilamide. Also the acetyl derivatives of sulfanilamide, sulfa-

* National Institute of Health, Bethesda, Md.

† Department of Medicine, Washington University, St. Louis, Mo.

1 Davis, B. D., *Science*, 1942, **95**, 78.

2 Woods, D. D., *Brit. J. Exp. Pathol.*, 1940, **21**, 74.

3 Wyss, O., Grubaugh, K. K., and Schmelkes, F. C., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 618.

4 Wood, W. B., Jr., *J. Exp. Med.*, 1942, **75**, 369.

5 Wood, W. B., Jr., and Austrian, R., *J. Exp. Med.*, 1942, **75**, 383.

6 Davis, B. D., in press.

TABLE I.
Binding of Sulfonamide Drugs to Plasma at pH 7.4.

Drug	Con. (M. × 10⁻⁴) of drug in buffer	Con. (M. × 10⁻⁴) of drug in plasma	Conc. protein (g%)	% of free drug bound per g protein	Avg min. bacteriostatic conc. (M. × 10⁻⁵)
Sulfanilamide	1.01	1.16	5.96	3.2	40
	1.85	2.13	5.38	3.5	
	1.84	2.12	5.63	3.3	
Sulfacetamide	0.98	1.19	5.45	4.6	20
	1.86	2.16	5.68	3.5	
	1.84	2.13	5.70	3.3	
Sulfaguanidine	0.60	0.75	5.90	5.1	20
	1.75	2.15	5.22	5.2	
	1.73	2.08	5.56	4.3	
Sulfapyridine	0.55	0.80	5.07	9.7	2
	1.5	2.5	5.21	13.6	
	4.7	6.9	5.16	10.0	
	10.0	14.4	5.18	9.3	
	19.6	26.0	4.89	8.2	
Sulfapyrazine	0.48	1.08	5.94	22.0	2
	1.5	2.7	6.22	14.0	
	1.55	3.40	5.21	24.0	
	3.0	5.1	5.50	14.0	
Sulfadiazine	0.50	1.03	5.11	22.0	2
	1.75	2.95	5.38	14.0	
	2.9	4.8	5.07	14.0	
	8.9	13.2	5.59	9.5	
	20.4	31.8	4.71	13.0	
Sulfathiazole	0.48	1.58	5.31	44.0	0.8
	1.50	4.6	5.45	39.0	
	4.4	10.6	5.72	25.0	
	8.7	18.0	4.76	23.0	
	18.0	34.2	5.44	17.0	

pyridine, sulfadiazine, and sulfathiazole, which are without bacteriostatic activity, are nevertheless bound to approximately the same degree as the corresponding unacetylated drugs.

Discussion. In the case of 7 of the most widely employed sulfonamide drugs there has been demonstrated a close correlation between bacteriostatic potency and the tendency to bind plasma protein. That the bacteriostatic activity of each drug is not determined by its power to bind protein is indicated by the fact that bacteriostatically inactive sulfonamide derivatives show the same tendency to bind protein. Data are presented by one of us[6] to support the conclusion that the dissociated anion of the sulfonamide compounds is essentially the form which becomes bound. On this basis the correlation of binding tendency with bacteriostatic potency can be adequately explained, for Schmelkes *et al.*,[7] and Fox and Rose[8] have recently demonstrated a striking parallelism between the acid-base dissociation constants and the bacteriostatic activities of a series of sulfonamide drugs. If only charged sulfonamide ions are bound to protein, the binding tendency of a given drug would be expected to be proportional to its dissociation constant and thus to its bacteriostatic activity. The fact that bacteriostatically inactive sulfonamide derivatives likewise bind protein is not inconsistent with this hypothesis, for there is conclusive evidence that the antibacterial properties of the sulfonamide drugs depend

[7] Schmelkes, F. C., Wyss, O., Marks, N. C., Ludwig, B. J., and Strandskov, F. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 145.

[8] Fox, C. L., Jr., and Rose, H. M., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 142.

TABLE II.
Binding of Related Compounds to Plasma at pH 7.4.

Drug	Conc. (M. × 10-4) of drug: buffer	Conc. (M. × 10-4) of drug: plasma	Conc. protein (g%)	% free drug bound per g protein
P-amino benzoic acid	0.55	0.70	5.71	5.4
	1.60	1.75	6.11	2.3
Sulfanilic acid	0.87	0.96	(Assume	2.5
	1.60	1.75	5.5)	2.3
Orthonilamide	0.55	0.80	5.12	9.6
	1.65	2.50	6.14	9.0
Metanilamide	0.58	0.68	5.44	3.9
	1.75	2.10	5.52	4.4
Acetyl sulfanilamide	5.1	7.0	5.45	7.5
" sulfapyridine	3.4	7.0	5.37	20.0
" sulfadiazine	3.0	5.4	5.62	15.0
" sulfathiazole	1.1	5.0	5.37	65.0

not only upon molecular dissociation but also upon the presence of the proper structural relationship in the dissociated anion.[2]

Summary. The protein-binding power of 7 commonly employed sulfonamide drugs has been measured by dialysis experiments. A quantitative correlation has been demonstrated between the protein-binding tendency and the bacteriostatic activity of the 7 drugs. Alteration of the p-amino structure of the compounds failed to affect significantly the protein-binding power although it destroyed the antibacterial properties. The data presented here and in another publication by one of us support the recently advanced hypothesis that the anionic species of the sulfonamide molecule is the active component involved in the mechanism of bacteriostasis.

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Action of Bacterial Toxins on Tumors. II. Effect of Sulfanilamide on Toxin-Induced Hemorrhage.

PAUL A. ZAHL AND S. H. HUTNER.
From The Haskins Laboratory, New York City.

It has been pointed out[1] that the lethal action of *Salmonella* endotoxin is markedly reduced *in vivo* by the administration of various sulfanilamide compounds. This lethal factor parallels in action the hemorrhagic factor present in the endotoxins of most gram-negative bacteria.[2] The parallelism of action in respect to sulfanilamide, as indicated by the following experiments, suggests the possibility that the two factors are similar, if not identical.

The toxic material used in this study was made by growing *Salmonella typhimurium* in a synthetic liquid medium. The methods for extraction have been previously described.[1] A single uniform batch of antigenic material representing the growth from forty liters of medium was used in both this and the previous study.[1] Observations of hemorrhagic effect were made on 7-day-old implanted tumors (Mouse Sarcoma 180).

Rockland mice bearing tumors were given

1 Hutner, S. H., and Zahl, P. A., *Science*, in press.

2 Zahl, P. A., Hutner, S. H., Spitz, S., Sugiura, K., and Cooper, F. S., *Am. J. Hyg.*, 1942, **36**, 224.

TABLE I.

Effect of Orally Administered Sulfanilamide on the Tumor-Hemorrhage Action of *Salmonella* Endotoxin.

Amt of toxin (μg intraperitoneal)	Amt of sulfanilamide (mg oral)	Degree of tumor hemorrhage				Total No. animals
		—	+	++	+++	
1300	0			3	9	12
1300	20			5	11	16
665	0			4	7	11
665	20	1		6	6	13
530	0			2	8	10
530	20			4	9	13
265	0			2	7	9
265	20		1	2	10	13
165	0			2	12	14
165	20		9	2	4	15
100	0		4	3	24	31
100	20	25	7	4	8	44
66	0		2	4	15	21
66	20	16	2	5	6	29
33	0	2	6	4	10	22
33	20	19	6	2		27
12.8	0	2	1	6	1	10
6.4	0	6	2	3	3	14
1.6	0	6	1			7

20 mg of neutralized sulfanilamide by stomach tube, followed by the intraperitoneal administration of the toxin in aqueous solution. Six to 10 hr after treatment the tumors were examined for hemorrhage.

From the table it is seen that the minimum *hemorrhage* dose is about 6.5 μg of dry weight of toxic material.* The minimum hemorrhage dose (M.H.D.) is defined as the smallest quantity of antigen which, when injected intraperitoneally, causes from slight (+) to marked (+++) hemorrhage in the tumors of 50% of the tested animals.

The minimum *lethal* dose of the endotoxin preparation used in this and the previous study[1] was approximately 1.3 mg for non-tumor-bearing mice. When this amount of the material was injected into tumor-bearing mice protected against death by adequate sulfanilamide treatment, marked hemorrhage occurred in the tumor, indicating that sulfanilamide does not negate the hemorrhage factor of the antigen at this dosage of the antigen. It is seen in the table that as the dosage of endotoxin was reduced respectively to 665, 530 and 265 μg, sulfanilamide remained ineffective so far as obviating the hemorrhage. At 165 μg, however, a suppression of hemorrhage by the sulfanilamide was observable; became more pronounced at 100 μg; and was obvious at 66 and 33 μg. Thus it would appear that sulfanilamide can substantially abolish the hemorrhage effect of no more than 16 minimum hemorrhage doses (selecting the hemorrhage dose represented by 100 μg).

It is of interest to note that in our previous study on the lethality of the endotoxin[1] it was found that sulfanilamide protected

* During the course of this study a highly purified hemorrhage-producing material was extracted from *Salmonella typhimurium* by the use of anhydrous phenol followed by fractionation of the complex toxic antigen by the acid-formamide procedure of Morgan and Partridge. The minimum hemorrhage dose of this purified material is between .05 and .10 μg. Experiments indicate that sulfanilamide exerts the same anti-hemorrhagic effect on this highly purified toxin at low doses as on the toxin preparation used in the present study.

against from 2 to 10 minimum lethal doses, a sign that the protection afforded by sulfanilamide against the hemorrhage factor parallels the protection afforded against the lethal factor. In protecting against lethality, sulfanilamide nullified the effect of 2.6 mg (2 M.L.D.) to 13 mg (10 M.L.D.).

However, in protecting against hemorrhage, sulfanilamide suppressed the effect of no more than 0.006 mg (about 1 M.H.D.) to 0.1 mg (about 16 M.H.D.). The ratio of M.L.D. to M.H.D. is about 1 : 200, obtained by dividing 1.3 mg (1 M.L.D.) by 0.006 mg (1 M.H.D.). Considering that only one two-hundredth of a lethal dose of the relatively unpurified toxin is required for tumor hemorrhage, an inhibition of, say, 90% of one M.L.D. of the toxin should leave a residual toxicity equivalent to 20 minimum hemorrhage doses. As seen in the table, animals may understandably be protected against the lethal effect of the toxin and yet undergo tumor hemorrhage. This observation constitutes further evidence that the vascular bed of the tumor, whose breakdown is responsible for the hemorrhage, is many times more susceptible to the toxin than is the organism as a whole to the lethal action of the toxin. Shear,[3] using a highly purified bacterial toxin, has reported that the hemorrhage-lethality ratio may be as high as 1 : 5000 if lethality is measured with non-tumor-bearing mice, and 1 : 500 if lethality is measured with tumor-bearing mice.

The mode of action of sulfanilamide in respect to protection against lethality and tumor-hemorrhage is as yet unknown. It is interesting that Andervont and Shimkin[4] found that ascorbic acid affords protection against toxin-induced tumor hemorrhage. It appears difficult to decide, however, whether this effect is mediated by raising the threshold of capillary resistance, or whether ascorbic acid (or sulfanilamide) may participate in a specific detoxication directed against the bacterial endotoxin.

In view of the previous conclusion[2] that the hemorrhage factor is a component of the O antigens of most gram negative bacteria, and in line with the evidence that sulfanilamide inhibits both the lethal effect and the tumor hemorrhage effect of these antigens, it appears reasonable from this parallelism in action that both tumor-hemorrhage and lethality are expressions of a common chemical factor characteristic of the O antigens.

3 Shear, H. J., *Cancer Research*, 1941, **1**, 731.

4 Andervont, H. B., and Shimkin, M. B., *Am. J. Cancer*, 1939, **36**, 451.

13940 P

Effect of Electrolytes upon Kahn Precipitates from Human and Animal Sera.

MORRIS N. GREEN AND HOWARD J. SHAUGHNESSY.

From the Division of Laboratories, Illinois Department of Public Health, Chicago, Illinois.

The problems created by the existence of falsely positive serologic reactions have been a source of difficulty since the advent of the Wassermann test.[1] Kahn[2] reported the first satisfactory differential test; this was based upon specific temperature differences.

We became interested in determining whether or not reactions in human and animal sera could be distinguished by the effect of electrolytes. Animal sera were used in these studies because they give general biologic reactions which are apparently of the same type as those found in human falsely positive serologic reactions.[2,3]

1 Wassermann, A., Neisser, A., and Bruck, C., *Deutsch med. Wchnschr.*, 1906, **32**, 745.

2 Kahn, R. L., *Arch. Derm. and Syph.*, 1940, **41**, 817.

3 Eagle, H., *Laboratory Diagnosis of Syphilis*, Chap. XVIII, C. V. Mosby Co., 1937.

While studying the effect of NaCl solutions upon the Kahn precipitates from animal sera, it was observed that the precipitate resulting from the reaction of Kahn antigen and horse serum was soluble in 4% NaCl solution. At the same salt concentration, precipitates from syphilitic human sera remain insoluble. The effect of other concentrations of NaCl (ranging from 0% to 30%) upon horse, human, guinea pig, hog, cow, sheep, and chicken sera were investigated. All sera were diluted to give approximately two plus reactions in the standard Kahn test by a method previously described.[4] Verification tests[2] were made upon each serum to check the specificity of the reaction. All sera gave the expected reactions (*i.e.*, either syphilitic, general biologic, or negative type) with this test. Controls were also made using the same quantities of serum and salt solution. However, an artificial antigen containing the same quantities of alcohol and saline as in the original antigen was used. Control reactions were completely negative except in the chicken sera.

It was found that each species of animal serum investigated gave characteristic aggregation and dispersion patterns of the Kahn precipitate. These were zonal in character. The most important differences in the behavior of the precipitates were as follows:

Sera from presumably non-syphilitic persons giving negative reactions in the standard Kahn test showed a complete lack of immediate precipitation. However, after standing overnight, strong precipitation occurred in the salt range from 0.0% to 0.3% similar to the results of Dunlop and Sugden,[5] and weak precipitation from 6% to 14% NaCl similar to Mackie and Anderson.[6] The reactions in other zones were completely negative. Syphilitic sera, however, gave maximal precipitation between 2% and 8% NaCl and practically no precipitation between 20% and 25% NaCl; the reactions in the rest of the zones were moderate.

Although guinea pig sera give no reactions in the usual serologic tests,[3] an immediate precipitation occurred at low salt concentrations (0.0% to 0.3% NaCl).

The Kahn precipitates from horse sera were dispersed and almost completely dissolved when concentrations between 2% and 6% NaCl were employed. The reactions in the other zones were moderate or strongly positive.

Hog, cow, and sheep sera gave almost identical reactions with this method. There was considerable variation in the results with individual sera. In general, however, strong reactions were obtained at low salt concentrations up to 0.3% There was a gradual weakening of precipitation until approximately 10 to 14% NaCl concentrations were reached. Very weak or negative reactions were then obtained. Moderate precipitation was again evident at about 27% salt concentration. The zone of negativity occurred at a much higher salt concentration in this group than in horse sera.

Chicken sera gave atypically coarse flocculation and very dense precipitation at high salt concentrations (25% to 30%). The controls gave results similar to the reactions with Kahn antigen. These precipitates were qualitatively and quantitatively different from the precipitates in the mammalian sera tested.

This is the first instance, as far as we are aware, in which the principle of differential electrolyte concentration has been used to distinguish false reactions in serodiagnostic tests from syphilitic reactions.* Aside from its bearing upon serologic tests, this method may be another means of distinguishing between the sera of some animal species and also determining additional biological rela-

[4] Green, M. N., and Forster, G. F., *Am. J. Syph., Gon. and Ven. Dis.*, 1942, **26**, 632.

[5] Dunlop, E. M., and Sugden, S., *J. Path. Bact.*, 1934, **39**, 149.

[6] Mackie, T. J., and Anderson, C. G., *J. Path. Bact.*, 1937, **44**, 603.

* Since this work was completed, Kahn[7] has announced a new practical verification test for distinguishing human syphilitic and human falsely reacting sera by a differential electrolyte concentration method applied to both serum and antigen suspension.

[7] Kahn, R. L., *Univ. Hosp. Bull.*, Ann Arbor, 1942, **8**, 45.

tionships between animal sera. Further studies are in progress in this field.

It was also observed that salt concentrations between 2% and 8% NaCl stabilized the Kahn reaction obtained with syphilitic sera by preventing fading. The sensitivity of these reactions was increased from 2 to 4 times that of the standard Kahn reaction in which 0.9% NaCl is used. The practical application of this finding is being further investigated.†

† We desire to express our appreciation to Dr. R. L. Kahn, University of Michigan, and Dr. J. Zichis of this laboratory, for their interest and criticism throughout the course of this investigation; also to Dr. E. J. Czarnetzky of Wilson and Company for his cooperation in furnishing many of the animal sera used in this study.

13941

Selective Filtration of Vitamin C by the Placenta.

ELLEN MCDEVITT, MARGARET A. DOVE, ROBERT F. DOVE AND IRVING S. WRIGHT.

From the Bonne Bay Cottage Hospital, Department of Health and Welfare, Norris Point, Newfoundland, and the Department of Medicine, New York Post-Graduate Medical School and Hospital, Columbia University, New York.

That the concentration of vitamin C is higher in cord than in maternal blood has been established.[1] The mechanism responsible for this phenomenon has been under dispute. Giroud[2] and his coworkers attributed it to the ability of the fetus to synthesize vitamin C. Others held that the depressing effects of exercise and sweating of labor[3] and of anesthesia, lead to the loss of vitamin C by the maternal organism. Manahan and Eastman[4] have established that this discrepancy can be explained by the selective action of the placenta.

We are presenting herein studies of 7 patients observed in the ante-natal clinic and the obstetrical ward of the Bonne Bay Cottage Hospital of Norris Point, Newfoundland. These patients were encountered in the course of a nutritional survey, a comprehensive report of which is in preparation.

Method. Blood plasma vitamin C determinations were carried out on nonfasting bloods, following the technic of Farmer and Abt.[5] Dietary histories were obtained to establish types of food ingested though no attempt was made to estimate caloric intake. Maternal vitamin C status was established before delivery by repeated blood plasma determinations. In 5 instances a sample of blood was obtained just prior to delivery. In 6 of the 7 patients, blood samples were also taken within 6 hr after parturition. Blood was collected from the umbilical stump in each instance at the time of delivery. All readings were made in triplicate.

Results. In all 7 instances, the vitamin C levels in the cord blood plasma were higher than in the maternal blood. As noted in Table I, patients No. 1 and 3, who had been receiving vitamin C therapy and whose blood levels were in the zone indicating saturation, showed proportional increase in vitamin C levels of the cord. Although patient No. 2 had been on a diet low in vitamin C, the cord blood was at the level of saturation. Patients No. 4 and 5 had been on diets in which the vitamin C content was negligible. The readings of the cord blood levels of patient No. 4 suggest the possibility of latent scurvy at birth, contrary to the implication of Braestrup.[1] Patient No. 5 was given one gram

1 Baestrup, P. W., *Acta Pediat.*, 1937, **2**, 328.

2 Giroud, A., *Compt. Rend. Soc. de Biol.*, 1936, **121**, 1062.

3 Smith, S., *The Vitamins*, Am. Med. Assn., 1939, **21**, 389.

4 Manahan, C. P., and Eastman, N. J., *Johns Hopkins Hosp. Bull.*, 1938, **62**, 478.

5 Farmer, C. J., and Abt, A. F., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 146.

TABLE I.

Patient 1		Patient 2		Patient 3		Patient 4		Patient 5		Patient 6		Patient 7	
Date	Vit. C mg%	Date	Vit. C mg%	Date	Vit. C mg%	Date	Vit. C mg%	Date	Vit. C mg%	Date	Vit. C mg%	Date	Vit. C mg%
1/31	0.31	12/ 4	.60	11/ 6	.39	3/31	.13	4/25	.13	10/10	.84	5/14	.31
2/11	0.87	18	.63	27	.62	4/16	.18	5/ 1	.18	11/20	.78	29	.31
21	1.10	1/ 8	.36	12/11	.60	20	.06			12/11	.89	6/ 3	.56
		22	.23	1/15	.00	23	.06			1/22	.69		
		2/ 5	.37	22	.17					2/12	.50		
		12	.31	2/ 6	.34					4/ 2	.37		
		19	.33							5/11	.43		
2/21/42		2/21/42		2/14/42 10 A.M.		4/24/42		5/1/42		5/15/42		6/10/42	
Cord Blood	1.90		1.30	Ante-Natal	1.43	Cord Blood	.33		.68		1.32		.93
				Cord Blood	2.21								
Post-Natal	0.63		0.27	3 P.M.	1.75		.06		.19		0.48		.33
Vit. C therapy begun 2/2/42. 12000 mg divided doses.				Vit. 2/6/42. 3400 mg divided doses				1 g crystalline Vit. C for saturation test 4/25/42. 225.4 mg returned in 5 hr.					

of crystalline vitamin C during a 5-hr saturation test which, while not changing appreciably the maternal blood level, probably contributed to the elevation of the cord blood in this instance. Patients No. 6 and 7 were on diets containing amounts of vitamin C above the average for the community.

Discussion. Since the maternal blood levels established before delivery by repeated determinations were essentially unchanged at delivery, it is evident that the discrepancy between the maternal and the cord bloods is not due to the depression of the maternal level by the exercise of labor or anesthesia.

The results of these studies demonstrate that the vitamin C supply for the fetus is secured by the placenta. Patient No. 4 is of particular interest since, with an extremely low maternal blood level (0.06 mg %) based on prolonged deprivation, the level of vitamin C in the cord blood was found to be elevated to only 0.33 mg %. In locales where adequate intake of vitamin C is impossible over long periods of time, it is important that some means should be undertaken to supplement the maternal dietary regime. The problem of latent scurvy at birth must be considered a very real one in many countries today.

Summary. 1. Additional data are presented to demonstrate the selective action of the placenta in filtering vitamin C from the maternal body. 2. Although this selectivity tends to take place at all levels of deficiency or saturation, latent scurvy may be present at birth, as the level of vitamin C in the placenta is, in the final analysis, dependent upon the maternal reserves of this substance.

The vitamin C used in these studies was Cebione, supplied through the courtesy of Merck and Company, Rahway, New Jersey.

13942

Pantothenic Acid Content of Pollen.*

PAUL B. PEARSON.

From the Department of Animal Husbandry and the Agricultural Experiment Station, Agricultural and Mechanical College of Texas, College Station.

While data are available on the thiamin[1] and riboflavin[2] content of bee bread, information on the distribution of members of the B vitamins in pollen is meagre. The report by Pearson and Burgin[3] that royal jelly is the richest known source of pantothenic acid suggested the possibility that this vitamin might be a factor in the development of the female larva of the honey bee *(Apis mellifera L.)* into queen bees. The queens and workers are produced from identical larvæ, as it is possible to rear queens from any female larvæ less than 3 days old by providing them with the proper food. For the first 2 days after hatching all female larvæ receive a diet of royal jelly. During the third day the diet of the larvæ that are to become workers is changed to pollen while the queen caste continues to receive royal jelly. In view of this difference in the diets between the two castes it appeared important to secure information on the pantothenic acid content of pollen.

Twenty-five samples of pollen were collected in various sections of Arkansas and Texas.† It was secured by means of pollen traps similar to the one described by Todd and Bishop.[4] Most of the samples of pollen were from more than one species of plant. Where the pollen was from a known species the analytical data are recorded separately in Table I. The pantothenic acid assays were made on the fresh pollen samples by the microbiological method of Pennington *et al.*[5]

A preliminary trial showed that enzymatic treatment of pollen as described by Waisman *et al.*,[6] for animal tissues did not increase the pantothenic acid values. Seven samples of pollen after autoclaving were adjusted to a pH of 4.6 with sodium acetate buffer and filtered in a similar manner to that described by Wegner *et al.*,[7] for removing foreign growth stimulants. The pantothenic acid values of the pollens so treated were not significantly lower than when the assays were made on the unfiltered material. The values for the filtered and unfiltered samples run simultaneously did not differ more than 3%. The pantothenic acid values reported in Table I were determined on the autoclaved pollen according to the original method.[5] The moisture content of each sample of pollen was determined so as to permit expressing the pantothenic acid values on both the fresh and dry basis.

The data on the pantothenic acid and moisture content of pollen are recorded in Table I. Expressed on a dry basis the pantothenic acid content ranged from 14.7 μg to 59.2 μg per g of pollen with an average of 30.0 μg per g. The moisture content ranged from 8.7% to 35.3% with an average of 17.8%. It is entirely possible that the pantothenic acid content of pollen varies with the botanical species of plant from which it is obtained, soil and climatic con-

* Published with the approval of the Director of the Texas Agricultural Experiment Station as technical contribution No. 745.

1 Haydak, M. H., and Palmer, L. S., *J. Econ. Ent.*, 1940, **33**, 396.

2 Haydak, M. H., and Palmer, L. S., *J. Econ. Ent.*, 1941, **34**, 37.

3 Pearson, P. B., and Burgin, C. J., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 415.

† Acknowledgments are made to S. E. McGregor of the Bureau of Entomology and Plant Quarantine, U.S.D.A., for collecting the pollen.

4 Todd, F. E., and Bishop, R. K., *J. Econ. Ent.*, 1940, **33**, 866.

5 Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1940, **135**, 213.

6 Waisman, H. A., Henderson, L. M., McIntire, J. M., and Elvehjem, C. A., *J. Nutrition*, 1942, **23**, 239.

7 Wegner, M. I., Kemmerer, A. R., and Fraps, G. S., *J. Biol. Chem.*, in press.

TABLE I.
Pantothenic Acid Content of Pollen.

Plant source	No. of samples	Water		Pantothenic acid			
				Fresh		Dry	
		Avg %	Range %	Avg μg/g	Range μg/g	Avg μg/g	Range μg/g
Mixed and unidentified	19	16.3	8.7-35.3	22.1	12.7-34.0	26.9	14.7-52.6
Buckeye *(Ungnadia speciosa)*	2	21.9	10.8-33.0	34.5	30.0-39.0	45.9	33.6-58.2
Youpon *(Ilex vomitoria)*	2	31.5	29.0-33.5	35.0	29.0-42.0	51.4	43.6-59.2
Cherry laurel *(Prunus caroliniana)*	1	13.4	—	18.5	—	21.3	—
Willow (species unidentified)	1	13.8	—	18.8	—	21.8	—
Avg	25	17.8		24.4		30.3	

ditions. This might account for the variation in the amount of pantothenic acid in various samples of pollen. Evidence for a species difference is afforded by the pantothenic acid values for the Buckeye and Youpon pollens which are considerably higher than the Cherry laurel, Willow or the unidentified species. It is of interest to note that the pantothenic acid content of pollen is of the same order as that reported by Jukes[8] for various plant materials. He reported values expressed in μg per g of dry material as follows: broccoli 46, kale 30, alfalfa 25, and spinach 10.

The pantothenic acid content of royal jelly is 511 μg per g of dry material.[3‡] Thus royal jelly contains about 17 times as much pantothenic acid as the average for pollen. This indicates that the honey-bee either has the ability to synthesize pantothenic acid or that there is a marked concentration of the vitamin which is transmitted to the royal jelly. Of the B vitamins thus far studied, pantothenic acid is the only one in which the amounts in royal jelly and pollen are not of approximately the same order. The universal biological importance of panthothenic acid has been reviewed by Williams[9] and from this it is evident that this vitamin is required by both the lower and higher forms of animal life. Unless it is subsequently established that the honey-bee is unique in being able to synthesize pantothenic acid, further support is afforded for the suggestion that this vitamin may be one of the factors responsible for the phenomenal properties of royal jelly in developing female larvæ into queen bees. When each of these factors has been individually determined and isolated it may then be possible to develop queen bees by artificial feeding.

Summary. Pollen samples have been assayed for pantothenic acid and found to contain an average of 30.3 μg per g on a dry basis. There is evidence that the pantothenic acid content of pollen varies with different plant species.

[8] Jukes, T. H., *J. Nutrition*, 1941, **21**, 193.

‡ Haydak and Palmer (*J. Econ. Ent.*, 1942, **35**, 319) reported that royal jelly contains 0.68 to 0.78 μg of pantothenic acid per g. Recently Palmer advised the author by personal communication that fresh samples of royal jelly had assayed over 500 μg per g on a dry basis.

[9] Williams, R. J., *Biol. Rev.*, 1941, **16**, 49.

13943 P

Effect of Ethanolamine and Betaine on Perosis in Chicks.*

JAMES MCGINNIS, L. C. NORRIS AND G. F. HEUSER.

From the School of Nutrition and Department of Poultry Husbandry, Cornell University, Ithaca, N.Y.

In the prevention of perosis in chicks and turkey poults it has been shown that manganese,[1] choline,[2,3] and biotin[4,5] are required. Stetten[6] has shown that in the rat ethanolamine and betaine serve as precursors in the biological synthesis of choline. Other studies[7] show that betaine can effectively replace choline as a donor of methyl groups for use in biological methylations. Jukes[8] has reported that betaine is ineffective in preventing perosis or promoting growth in turkey poults. Ethanolamine, however, was not fed in the experiments conducted by Jukes. In view of this, experiments were conducted to determine the effect of ethanolamine and betaine in preventing perosis in chicks.

Day-old Rhode Island Red cockerel chicks were divided into 4 groups of 20 chicks each and placed in battery brooders with wire floors. The experimental diet was supplied immediately and fed *ad libitum* thereafter. Individual weights were recorded at weekly intervals at which time any symptoms of perosis were noted. The method used in obtaining the severity index was that described by Wilgus, Norris and Heuser.[9] All experiments were terminated at the end of 6 weeks.

The basal diet fed in these studies contained 66.75% degerminated yellow corn meal, 15 peanut meal, 10 purified casein, 3 soybean oil, 0.25 reinforced cod liver oil, and 5 salt mixture.[10] To each 100 g of the basal diet were added 300 μg of thiamin, 500 riboflavin, 500 pyridoxine and 700 d-calcium pantothenate. A new supply of corn meal, peanut meal and casein was used in preparing the basal diet fed in Experiment 2.

The results of the experiments in which ethanolamine and betaine were used as supplements to the basal diet are summarized in Table I.

In Experiment 1 ethanolamine was slightly effective in reducing the amount of perosis but the same results were not obtained in Experiment 2. No effect upon mortality was observed in either experiment whereas the influence upon growth was variable. The effectiveness of ethanolamine in Experiment 1 may have been due to the presence of more labile methyl groups in the ingredients used in preparing the basal diet fed in this experiment than were present in the ingredients used in Experiment 2.

Betaine, on the other hand, was effective in preventing perosis. It also promoted growth and markedly reduced the chick mortality. These results are not in accord with those obtained by Jukes[8] in experiments with turkey poults.

The addition of both ethanolamine and betaine to the basal diet was somewhat more effective in reducing perosis than betaine alone. This may have been caused by the biological synthesis of a greater amount of

* This work was made possible by the establishment of a Fellowship at Cornell University by the Grange League Federation Exchange, Inc., of Ithaca, N.Y.

1 Wilgus, H. S., Jr., Norris, L. C., and Heuser, G. F., *J. Nutrition*, 1937, **14**, 155.

2 Jukes, T. H., *J. Biol. Chem.*, 1940, **134**, 789.

3 Hogan, A. G., Richardson, L. R., Patrick, H., and Kempster, H. L., *J. Nutrition*, 1941, **21**, 327.

4 Jukes, T. H., and Bird, F. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 231.

5 Richardson, L. R., Hogan, A. G., and Miller, O. N., *Mo. Sta. Res. Bul.* 343, June, 1942.

6 Stetten, DeWitt, Jr., *J. Biol. Chem.*, 1941, **140**, 143.

7 Chandler, J. P., and du Vigneaud, V., *J. Biol. Chem.*, 1940, **135**, 223.

8 Jukes, T. H., *J. Nutrition*, 1940, **20**, 445.

9 Wilgus, H. S., Jr., Norris, L. C., and Heuser, G. F., *Poultry Science*, 1937, **16**, 232.

10 Schumacher, A. E., and Heuser, G. F., *Poultry Science*, 1940, **19**, 315.

TABLE I.
Effect of Ethanolamine and Betaine on Perosis.

Supplement to basal diet	Perosis		Avg wt at 6 wk, g	Mortality, %
	Incidence, %	Severity index		
Experiment 1.				
0	87.5	47.5	290 ± 51.8	40
.2% ethanolamine	54.0	28.6	304 ± 58.1	40
.2% betaine hydrochloride	22.2	6.8	396 ± 84.4	10
.2% betaine hydrochloride + 0.2% ethanolamine	10.5	2.6	440 ± 50.3	5
Experiment 2.				
0	87.0	46.0	314 ± 72.9	35
.2% ethanolamine	87.0	41.5	263 ± 33.5	40
.2% betaine hydrochloride	25.0	8.0	383 ± 80.2	0
.2% betaine hydrochloride + 0.2% ethanolamine	15.0	8.6	362 ± 69.2	10

choline in the presence of the added ethanolamine.

The effectiveness of betaine in preventing perosis was nearly as great, if not as great, as choline was found to be in an experiment started 2 weeks earlier than Experiment 1. In the choline experiment the chicks were identical in strain and source with those used in the experiments on betaine and the composition of the basal diet was also identical. The ingredients used in preparing the diet were the same as those used in Experiment 1 on betaine. The incidence of perosis in the choline experiment was 21.0%, the severity index 6.7, the average weight at 6 weeks of age 421 g and the mortality 5%.

Summary. The occurrence of perosis in chicks was markedly reduced by supplementing the basal diet with betaine hydrochloride. Ethanolamine was slightly effective in one experiment in preventing perosis but not in the other. Supplementing the diet with both ethanolamine and betaine was somewhat more effective in reducing perosis than either one alone. Under the experimental conditions betaine also promoted growth and reduced mortality.

13944

Effect of Hypophysectomy of Growing Chicks upon Their Basal Metabolism.*

A. NALBANDOV AND L. E. CARD. (Introduced by F. C. Koch.)
From the Department of Animal Husbandry, University of Illinois, Urbana.

In the course of a study dealing with certain fundamental aspects of physiology of reproduction in domestic chickens, young White and Brown Leghorn chicks were hypophysectomized. No reports of basal metabolic rates of hypophysectomized chickens were found in the literature and this fact, together with the finding of complete postoperative regression of the thyroids and apparent apathy of the birds prompted the study summarized in this report.

Since details of the experiments are to be reported elsewhere only an abridged statement of the experimental approach is given here. Both the operated and the normal birds were hatched on April 26, 1941. They were raised under standard laboratory conditions and were operated upon when weighing about 500 g, at an age ranging between 46 and 51 days.

Following the removal of the pituitary

* We are indebted to Dr. T. S. Hamilton and Mr. W. T. Haines for their valuable help and suggestions.

TABLE I.
Average Basal Metabolic Rates of Normal, Hypophysectomized and Starved Chickens.

Item	Animal No.	Sex	Treatment	Body wt at 48 days (g)*	Body wt at 232 days (g)†	Respiratory quotient	Heat production Per M² body surface	Heat production Per kg body wt
1	3838	M	Normal	423	2,000	.736	83.65	41.83
	3807	M	"	448	2,040	.667	86.74	42.52
2	3880	M	Castrate	500	2,076	.702	79.84	38.46
	3806	M	"	450	2,367	.693	88.74	37.49
3	3829	M	Incompletely hypophysectomized	475	1,280	.723	57.82	45.17
4	3872	M	Completely	513	1,353	.703	45.14	33.36
	3794	M	hypophys.	487	1,310	.856	44.69	34.11
5	3825	F	Normal	402	1,252	.694	84.83	67.76
	3826	F	"	452	1,500	.701	90.02	60.01
6	3878	F	Incompletely hypophys.	615	958	.677	56.64	59.12
7	3131	F	Completely	405	1,352	.712	56.58	41.85
	3113	F	hypophys.	458	1,203	.760	50.42	41.91
8	3838	M	Normal; on restricted feed intake	423	1,630	.726	60.15	36.90
	3807	M		448	1,765	.737	59.05	33.44

*Average age at hypophysectomy.
†Average age at BMR tests.

gland most birds recovered rapidly and a few lived for as long as 6-12 months. The voluntary food intake of operated birds was 30-50% lower than that of normal birds but in spite of this most of the hypophysectomized birds continued to gain in weight. This was found to be due to an unusual adiposity of all tissues rather than increase in length of the longbones and the skeletal tissues as a whole. Complete or nearly complete hypophysectomy also resulted in marked apathy of all birds, and pronounced changes in plumage pattern and texture. Upon autopsy the birds were found to have microsplanchnia, greatly enlarged gall-bladders, macro- and microscopic regression of all glands dependent on secretions from the pituitary gland.

Experimental. All determinations of the basal heat production were made at room temperature, care being taken that the temperature did not exceed the thermo-critical range of the birds. Since hypophysectomized animals in general are supposed to be subject to hypoglycemic shock when fasted, and since hypophysectomized chickens in particular were found easily affected by any adverse environmental conditions, it was decided to fast both operated and normal chickens for only 24 hr prior to the test and compare the results obtained with BMR determinations made on normal birds fasted for 48 hr. The test itself was run over a period of 23 hr and a Haldane type gravimetric respiration apparatus as described and used for chickens by Mitchell and Haines[1] was employed.

Each bird was subjected to 2 tests at intervals of 2 weeks and as the variation in all cases but 2 did not exceed 5%, the two tests were combined.

At the time of the BMR determinations, the completeness of the operation was judged by the condition and the color of the plumage and particularly the size of the head furnishings and the other secondary sex characters. However, all the birds eventually died or were killed and the completeness of the operation verified by sectioning of the hypophyseal region.

Results. Inspection of the tabulated data shows that complete hypophysectomy reduced basal heat production in males to 80% (items 1 and 4, Table I) and in females to

[1] Mitchell, H. H., and Haines, W. T., *J. Agr. Res.*, 1927, **34**, 549.

60% of normal (items 5 and 7, Table I). Riddle, Smith, and Moran[2] have shown that complete or nearly complete absence of the pituitary gland in pigeons lowered basal metabolism 40-50% when the determinations were made at the animals' own critical temperature. Similar results have been reported for mammals.

Hamilton[3] has shown that the basal heat production of rats may be significantly lowered by restricting their food intake. Since removal of the pituitary decreased consumption of food by the birds by 30-50%, it was suggested that the reduction of the BMR of operated birds may be partially or totally due to decreased food consumption rather than hypopituitarism. To test this possibility, 2 normal male hatch mates (Nos. 3807 and 3838) of 2 hypophysectomized males (Nos. 3872 and 3794) which were previously fed *ad libitum* were restricted to the same food intake as was voluntarily consumed by the 2 birds lacking the pituitary. After 25 days of restricted feeding, the normal males had stabilized their body weights at about 400 g lower than their normal previous weights. The "starved weights" of these 2 birds were still 400 g higher than the weights of the hypophysectomized birds. After 30 days' restricted feeding the BMR's of the normal and operated birds were run and repeated 7 days later. Again the variation between the two tests did not exceed 5% and they were combined. It was found that restricted food intake alone had lowered the BMR of normal birds to about the level of the BMR of completely hypophysectomized males when both are expressed in comparable terms of heat production per kg of body weight. (Items 8, 4, and 1 in Table I). Placed again on full feed, the normal males regained and exceeded their normal level of basal heat production within a few days.

While the cause and effect relationship between hypopituitarism and BMR, and level of food intake and BMR have not been disentangled by the above experimental approach, it appears that the level of food intake is of importance as a regulatory mechanism of basal heat production of both normal and pituitary-less chickens. The postoperative depressions of the BMR may be due to decreased food intake but in normal birds, however, restricted diet is not necessarily the primary cause of depressed BMR because the possibility of a hypopituitary condition caused by starvation has not been ruled out in the above experiment.

Summary. Ablation of the pituitary gland of immature chickens results in a depression of the basal heat production, in males to 80%, in females to 60% of normal. Hypophysectomy results in a voluntary food consumption of one-third to one-half of normal. Restriction of the food intake of normal birds to the level of hypophysectomized birds results in a depression of the BMR of normal birds to about the level of their operated controls. These results suggest that basal heat production may be directly influenced by the level of food intake rather than by hypopituitarism. The possibility of indirect effect of restricted food intake has not been ruled out.

[2] Riddle, O., Smith, G. C., and Moran, C. S., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1614.

[3] Hamilton, T. S., Ph.D. Thesis, U. of Ill., Urbana, 1937.

13945 P

Reproduction of Bacteria from the Large Bodies of *B. funduliformis*.*†

L. Dienes and William E. Smith.

From the Department of Pathology and Bacteriology, the Massachusetts General Hospital, and the Robert W. Lovett Memorial Foundation, Harvard Medical School, Boston, Mass.

The significance of large round bodies which develop in various bacterial cultures was discussed in a preceding paper.[1] The germination of the large bodies usually produces a peculiar growth which in appearance and morphology is similar to the pleuropneumonia group of organisms. Observations of certain cultures suggest that this is not the only way in which the large bodies germinate; they may reproduce also bacteria of usual shape and size. The development of bacteria inside the large bodies was clearly visible in one instance in a colon bacillus culture.[2] The observation of a *B. funduliformis* strain in which this process occurred regularly and could be reproduced at will allowed a more thorough study of it.

Transformation of bacteria into large swollen forms is a characteristic property of *B. funduliformis*. In the first strain which was studied, the germination of the large bodies produced pleuropneumonia-like colonies which could be propagated in pure culture. In a second strain, under certain conditions, all bacteria swell up to large bodies, but these gave only bacterial growth in transplants. The method of derivation of the bacteria from the large bodies was not definitely established, but preparations made a few hours after transplantation suggested that the bacteria developed inside of the large bodies and were freed by their disintegrations. A third strain presented special advantages for the study of the development of large bodies. The cultures of this strain on solid media consisted of small regular bacilli. In liquid media most of the bacilli swell up into large bodies. Bacillary forms were seen only rarely in broth cultures 36 hr old. After further incubation the large bodies disappeared and the culture consisted again of regular bacilli.

This strain grew very well on agar blocks under a coverslip surrounded by paraffin, anærobiosis being obtained by inoculating a corner of the agar with staphylococci. On such agar blocks inoculated with 36-hr broth cultures only the large bodies were visible during the first few hours of incubation. After 4 to 6 hr, pleuropneumonia-like growth started from some of the large bodies. These were less refractile than the others and in stained preparations remained paler. Most of the large bodies developed into bacterial colonies. This occurred in various ways. A small sized large body may elongate itself to bacillary form or take up an irregular angular shape which by division falls into bacilli. Some of the large bodies fractioned into 2 or 4 parts and these developed into regular bacteria. The majority of the large bodies developed in a different way. They increased first in size and density and after 5 to 6 hours' incubation developed many prominences, taking up an irregular mulberry-like shape. The prominences developed to bizarre-shaped branching filaments which by fractionation produced regular bacilli. The filaments started in many directions from the large body sometimes producing a polyp-like structure. In stained preparations it may often be seen that the mulberry shape is produced by the fractionation of the contents of the large body into granules. These granules grew out into the branching filaments. Occasionally regular shaped bacilli developed inside of the large bodies and were freed by their disintegration.

The mulberry-shaped structures consisting

* The expenses of this investigation have been defrayed in part by a grant from the Commonwealth Fund.

† This is publication No. 65 of the Robert W. Lovett Memorial Foundation for the study of crippling disease.

1 Dienes, L., *J. Bacteriology*, 1942, **44**, 37.

2 Dienes, L., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 773.

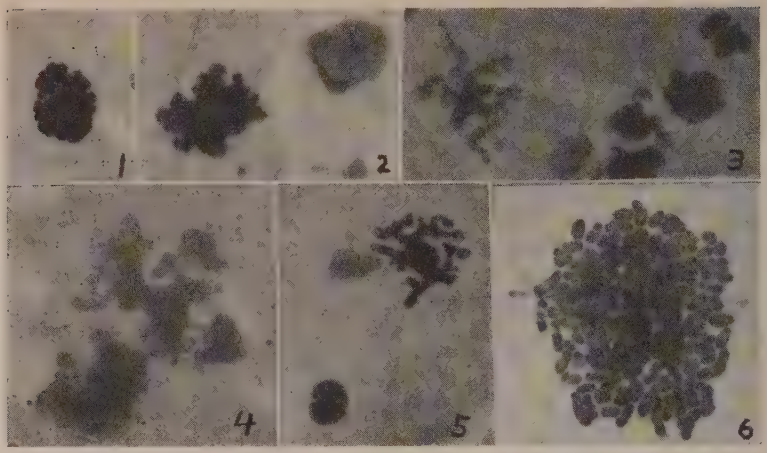

FIG. 1.

Photographs 1 to 6 show consecutive stages of the development from large bodies into bacterial colonies. Some of the large bodies in the photographs did not develop and show their original appearance. Photographs 1, 2, 3 and 5 were made from impression preparations stained with Giemsa following Klieneberger's[3] agar fixation technic; 4 was made from a stained agar block and 6 from an impression preparation of an agar block stained with safranine.

of granules which develop to branching filaments are in some respects similar to the pleuropneumonia-like growth, but the filaments do not grow into the agar and within a few hours develop into regular bacilli. These structures probably represent unstable transitional forms between the pleuropneumonia-like and the regular bacterial growth. The strain has a tendency to grow in both forms on solid media.

The successive appearance of large bodies and regular bacterial forms in the cultures of our second and third strains indicates that reproduction by means of the large bodies occurs regularly under certain conditions. The observation of a similar process in a colon bacillus culture already has been mentioned. It was claimed repeatedly in the literature that the large bodies were organs of reproduction but this conception did not gain recognition because the observations could not be reproduced at will and the pictures published were not regarded as decisive. The observations with our third strain seem to give definite evidence that the large bodies take part in the reproduction. The dual potentiality in the development of the large bodies remains an interesting problem.

[3] Klieneberger, E., and Smiles, J., *J. Hygiene*, 1942, **42**, 110

13946 P

Use of Mice in Testing of Antigenic Power of Tetanus Toxoid.

WALTER L. KOERBER AND GERTRUDE E. MOOK. (Introduced by W. E. Bunney.)
From the Biological Laboratories, E. R. Squibb and Sons, New Brunswick, N.J.

A simple and accurate method is herein described for testing the antigenicity of tetanus toxoid, using mice instead of guinea pigs. Preliminary experiments with Swiss mice have indicated that the following factors have to be considered in order that uniform results in repeated antigenicity tests may be obtained. (1) Source of mice. Antibody response varied according to source. (2) Route of immunization. Intraperitoneal route gave optimum response. (3) Sex of mice. Female mice showed better response than male. (4) Immunization period. An interval of 21 days between a single injection of toxoid and the test injection of 10 mouse MLD seemed to be the shortest time to insure reproducible results.

After due consideration of the above factors a tetanus toxoid, previously tested on guinea pigs was adopted as provisional standard for use in repeated tests for immunization of Swiss mice. The results of 4 tests show a high degree of uniformity. From 93 to 96% of mice immunized with a provisional standard toxoid resisted the test with 10 M.L.D. of toxin in 4 separate experiments involving a total of 251 mice. The effect of varying amounts of toxoid (made up to 1 ml with peptone diluent) on the antibody formation of Swiss mice of the same source can be seen in Table I. A detailed description of two alternative methods of practical application is given here.

Procedure I. Swiss mice from a constant source are standardized for their antigenic response by repeated tests using the provisional standard toxoid as immunizing agent. This standard toxoid may be either a liquid or dried toxoid of proven constant antigenicity. At least 20 female and 20 male mice of 18 to 20 g weight are injected intraperitoneally with 1 ml of the standard toxoid, rested for 21 days and exposed to a subcutaneous injection of 1 ml containing 10 Mouse MLD (= 2 Guinea Pig MLD) of an aged tetanus toxin of constant titer. Five non-immunized control mice are exposed to 1 Mouse MLD of toxin and should die within 96 hr. The exposed mice are observed for 7 days and the average number of protected males and females recorded. This figure represents the "standard value of protection" for the chosen strain of Swiss mice. This figure should be checked every few months. This value serves as comparator for testing toxoids of unknown antigenic titer which should show as good or better protection than the standard.

Procedure II. This method does not involve a standardization of mice from a known source. Each test of tetanus toxoid of unknown titer includes a control of mice which received an intraperitoneal injection of 1 ml of the standard toxoid. After 3 weeks the 2 groups of mice are challenged with 10 MLD, and the non-immunized mice with 1 Mouse MLD of toxin respectively. The protection resulting from the use of the

TABLE I.
Effect of Varying Amounts of Toxoid on Resistance to Toxin in Swiss Mice.

Amt of toxoid *A* inj., ml	Route of immunization	Immunization period, weeks	No. of mice exposed to 10 MLD toxin	Avg % protected
1.0	Intraperit.	3	41	95
0.5	"	3	42	70
0.25	"	3	41	30
1.0	Subcutan.	3	57	95
0.5	"	3	58	64
0.25	"	3	64	28

TABLE II.
Comparative Antigenicity Tests Using Mice and Guinea Pigs.

Toxoid	Mice			Guinea pigs		
	Immunization period, weeks	No. of animals tested	% protected	Immunization period, weeks	No. of animals tested	% protected
Stand. *A*	3	41	95	6	13	92
No. 1	3	38	96	6	8	87
	3	40	93			
No. 2	3	40	95	6	16	75
				6	10	90
No. 3	3	39	92	6	13	92
No. 4	2	69	100	6	12	92
No. 5	2	67	96	6	13	85
No. 6	3	40	77	6	10	40

unknown toxoid is then compared with that of the standard toxoid tested simultaneously. The technic of the test is otherwise the same as described under Procedure I.

Using Procedure I we have compared the degree of resistance attained in mice and guinea pigs and have obtained the data listed in Table II.

Summary. Swiss mice can be used as test animals in place of guinea pigs for the determination of the antigenic power of tetanus toxoid. The advantage in the use of mice lies in two facts: (1) the immunization period necessary is only 3 weeks instead of the usual 6 weeks; (2) lower cost of mice permits the use of a larger number of animals and animals of greater uniformity than guinea pigs, which contributes to a greater statistical significance and accuracy of the test.

13947 P

Activity of Desoxycorticosterone Acetate upon Oral Administration.

HEINZ FRAENKEL-CONRAT.*

From the Institute of Experimental Biology, University of California, Berkeley.

Comparative studies of the activity of desoxycorticosterone acetate upon parenteral and oral administration have been carried out repeatedly. Grollman found 1 mg of this hormone effective both in maintaining life and promoting growth of adrenalectomized rats, regardless of the mode of administration.[1] On the other hand, more recent studies indicated that the effects produced by injection of the hormone were considerably superior to those following oral administration.[2] A comparison of the technics of oral administration in the earlier and in the later studies indicated a difference which appeared of possible significance. Grollman mixed the hormone with the diet and thus ensured prolonged or almost continuous intake of the hormone. On the other hand, Kuizenga gave the hormone, dissolved

* Present address: Western Regional Research Laboratory, U.S. Department of Agriculture, Albany, Calif.

1 Grollman, A., *J. Pharm. and Exp. Therap.*, 1939, **67**, 257.

2 Kuizenga, M. H., Nelson, J. W., and Cartland, G. G., *Am. J. Physiol.*, 1940, **130**, 298.

TABLE I.

Exp. No.	Type of treatment	Daily dose, mg	No. of rats	20-day survival, %	Wt change (20 days) g	Survival (days)† Avg	Individual
1	Doca, oral	.09-0.8*	4	75	+22	1 rat died 3rd day*	
	Controls		6	0	—	6	2,2,2,7,9,15
2	Doca, oral	.35	7	29	+10	13	7,13,13,14,16
	Doca, parenteral	.33	3	67	+25	1 rat died 13th day	
	Controls		4	0	—	3	2,3,4,4

†Of the rats which died during the experimental period.

*During first 3 days hormone given only in drinking water, an average of only 0.09-0.16 mg being consumed daily by each rat; during next five days hormone also mixed with diet, 0.8 mg per day being consumed in average; during the rest of the experimental period the hormone was again given only in drinking water, an average of 0.26 mg being consumed by each rat.

in a small volume of oil, once daily by stomach tube.[2] It appeared possible that the absorption of the hormone might not be complete under these conditions. Evidence for the fact that the technic of oral administration may play a significant role in determining the availability of a substance has recently been demonstrated by Schoenheimer's studies.[3] In the case of desoxycorticosterone, recent studies of the effect of adrenal hormones on liver arginase activity indicated this hormone to be effective when given to hypophysectomized rats in solution in the drinking water.[4] It thus seemed indicated to reinvestigate in adrenalectomized rats the activity of desoxycorticosterone upon oral administration under physiological conditions, *i.e.*, by mixing the hormone with diet and drinking water.

Female rats were adrenalectomized at the age of 26-28 days.† They received 1% sodium chloride to drink for one week. From the eighth day on, all rats received a salt-free diet and no more salt water to drink. In Experiment 1, they were then divided into 2 groups, one receiving hormone and the other serving as control. The hormone was given in solution in the drinking water (0.0025 or 0.0033%) as well as, from the fourth to the eighth day, mixed with the diet. In Experiment 2, the operated rats were divided into 3 groups; one received the hormone (in solution in sesame oil twice daily) by subcutaneous injection, another received a similar dose mixed with drinking water (0.0025%) and diet and a third served as control. The hormone intake was determined for groups of 2 or 3 rats and thus represents an average figure. At the end of a 20 day period of treatment, hormone was withdrawn from all rats until death (1 to 9 days). At autopsy the lumbar region of all operated rats was searched for residual or accessory adrenal tissue. Suspicious tissue was fixed and studied histologically.[2] A few rats (9% of the total) showed weight gains which were far in excess of those of all other operated rats, treated as well as untreated, while no adrenal tissue could be found by binocular inspection at autopsy. These doubtful cases as well as those in which adrenal tissue was found were not included in the table.

Results. Orally administered desoxycorticosterone acetate was found to maintain the life of adrenalectomized rats and enable them to grow. In Experiment 1 (see Table I), as low a dose as 0.09 to 0.16 my daily led to a survival of 3 out of 4 rats during the first 3 days, while 3 of 6 controls died during this period. The 3 treated rats also survived and continued to grow throughout the rest of the experimental period, during which the daily hormone dosage was raised to 0.8 mg for 5 days and then again reduced to 0.26 mg. In Experiment 2, the daily oral administration of 0.35 mg hormone enabled all rats to sur-

[3] Schoenheimer, R., Ratner, S., Rittenberg, D., and Heidelberger, M., *J. Biol. Chem.*, 1942, **144**, 545.

[4] Fraenkel-Conrat, H., Simpson, M. E., and Evans, H. M., in press.

† The author is indebted to Dr. M. E. Simpson for her cooperation in performing the operations as well as the histological examinations of possible adrenal remnants.

vive the controls, although only 2 of the 7 rats of the treated group survived the entire experimental period. Gains in body weight were also less pronounced in this than in the corresponding group in Experiment 1. Comparison of oral and parenteral administration indicated that the latter was slightly superior. However, the number of completely adrenalectomized animals receiving the hormone by injection was too small to warrant foregoing quantitative conclusions in this regard (adrenal tissue was found in 3 rats of this group).

Summary. Desoxycorticosterone acetate was found to be beneficial to adrenalectomized rats upon oral administration, *i.e.*, mixed with food and/or drinking water. Dosages of 0.09 to 0.33 mg daily favored survival and growth in such rats on a salt-free diet.

13948 P

Partial Maintenance of Adrenal Weight in Hypophysectomized Immature Male Rats by Testosterone Injections.*

SAMUEL L. LEONARD.

From the Department of Zoology, Cornell University, Ithaca, N.Y.

In the course of certain experiments involving the use of hypophysectomized male rats, where routinely it has always been customary to weigh the adrenals, it was noted that after male hormone treatment, the adrenals did not regress to the same degree as did those of litter mate controls. After hypophysectomy, the adrenals always atrophy and lose weight[1] and this observation has been one of the criteria indicating completeness of the operation. Since no mention of a maintenance effect of male hormone on the adrenals of hypophysectomized rats has been made in the literature, a preliminary note on this finding is here presented.

All the rats used in these experiments were from the Long-Evans strain, between the ages of 22 and 35 days at the time of hypophysectomy. There were fed a dry ration of dog and calf meal,[2] water and a 25% solution of corn syrup *ad libitum*. A part of each litter of hypophysectomized males were injected once daily with .05 cc = 1.25 mg of testosterone propionate (Peranderen, Ciba)† for 10 days, the operated litter mate controls received no injections. At autopsy on the 11th day, the adrenals were removed, carefully trimmed of the surrounding fat and were weighed fresh. Some of the glands were prepared for histological study. Serial sections of all the sellæ turcicæ were made and all incompletely operated animals were discarded. In all, 8 litters, involving 22 injected and 14 control rats, were used.

TABLE I.

Testosterone Inhibition of the Adrenal Atrophy Which Follows Hypophysectomy.

Exp.	Age	Avg adrenal wt (mg) Injected	Controls	% Difference
I	34	10.4 (4)	8.5 (1)	22
II	35	17.5 (4)	9.5 (2)	84
III	24	11.9 (5)	7.4 (4)	60
IV	25	12.2 (4)	8.0 (1)	53
	22	11.6 (2)	6.4 (2)	81
V*	23	13.4 (3)	6.4 (4)	109

*Total dose of testosterone 6.9 mg in 10 days, all others received 12.5 mg.

Numbers in ( ) indicate number of animals. Results on litters of same age combined.

A comparison of the average adrenal weights of the injected and control rats within the separate litters is given in Table I, together with the percentage difference of the

*Aided by a grant from the Sackett Research Fund.

1 Smith, P. E., *Am. J. Anat.*, 1930, **45**, 205.

2 Maynard, L. A., *Science*, 1930, **71**, 192.

† Acknowledgment is made to Dr. R. MacBrayer of Ciba, Summit, N.J., for a generous supply of hormone.

averages. The latter varied from 22% to 109%. The individual adrenal weights of the injected rats were consistently heavier than the control weights within a given litter. Some atrophy of the adrenals of the injected rats did occur because glands from normal rats at the time of operation weighed from 16 to 20 mg, depending on the age of the rat. Most all of the injected rats gained in body weight or lost only a few grams. There did not seem to be any definite correlation between body weight changes and the degree of adrenal involution in these preliminary experiments. Histologically, the cortex appeared more normal in the injected rats, and the general appearance and yellowish color of the fresh adrenals were like that of normal rats.

Three similar experiments were carried out except that an interval of 7-9 days was allowed before beginning injections. The results were rather equivocal as adrenal weight differences in 3 litters of 5, 4 and 4 rats were respectively 0%, 33% and 56%. The adrenals of the injected rats were heavier than those of the controls in the two latter cases.

Male hormone substances have been reported not to affect the adrenals of hypophysectomized rats,[3] or mice[4] which is similar to the reported non-effect of progesterone[5] and cortical hormones.[6,7] Atwell[8] stated that the adrenal cortical cells of hypophysectomized larval *Rana sylvatica* were somewhat restored by cortical extract. In the annual breeding ground squirrel, the adrenal cortex hypertrophied along with sex organs on experimentally induced sexual recrudescence.[9]

Summary. Injections of large doses of male hormone partially prevent the atrophy of the adrenal cortex which normally follows hypophysectomy in the immature male rat.

[3] Walsh, E. L., Cuyler, W. K., and McCullagh, D. R., *Am. J. Physiol.*, 1934, **107**, 508.

[4] Nelson, W. O., and Merckel, C. E., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 737.

[5] Selye, H., *Anat. Rec.*, 1940, **78**, 253.

[6] Atwell, W. J., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 1259.

[7] Ingle, D. J., *Am. J. Physiol.*, 1938, **124**, 369.

[8] Atwell, W. J., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 621.

[9] Moore, C. R., Simmons, G. F., Wells, L. J., Zalesky, M., and Nelson, W. O., *Anat. Rec.*, 1934, **60**, 279.

13949

Nitrogen Balance in Human Tryptophane Deficiency

WARREN M. COX, JR., ARTHUR J. MUELLER AND DALLAS FICKAS.
From the Laboratories of Mead Johnson and Company.

Many reports on the inadequacy of tryptophane-deficient protein hydrolysates in animal nutrition are available. With the exception of the recent report of Holt and coworkers,[1] we have been unable to find any report of tryptophane deficiency in man.

In February, 1940, we had the opportunity of studying a patient with a gastrostomy. The patient, a 65-year-old woman with cancerous occlusion of the esophagus, had, of necessity, reduced her food intake progressively during the 6 months prior to admission to the hospital. Difficulty in swallowing was progressive. For the week preceding operation she had been unable to swallow any food by mouth; and could retain but little fluid. A gastrostomy opening was made under local anesthesia on January 27th and the second day thereafter studies on nitrogen balance were begun.

Because of the long-continued protein starvation, nitrogen was not given at a basal level. None the less, we hoped to make observations on tryptophane deficiency in a

[1] Holt, L. E., Jr., Albanese, A. A., Brumback, J. E., Jr., Kadji, C., and Wangerin, D. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 726.

TABLE I.

Nitrogen Balances on Milk, and Enzymic and Acid Hydrolysates of Casein.
Patient F.N. Weight 41 kg. Gastrostomy performed 1/27/40.
(Expressed as g per kilo per day.)

Period	Date	Supplement	Total nitrogen intake	Output		Balance	Plasma	
				Feces	Urine		Protein g %	Albumin g %
1	1/30/40	None	None	.009	.063	—.072		
2	31	Enzymic	.195	.008	.088	+.099	6.45	4.08
	2/1	Hydrolysate	.184	.050	.070	+.064		
	2		.197	.010	.109	+.078		
		Avg	.192	.023	.089	+.080		
3	3	Milk	.194	.036	.111	+.047	6.10	3.46
	4		.196	.012	.097	+.087		
	5		.199	.002	.127	+.070		
		Avg	.196	.017	.112	+.068		
4	6	Enzymic	.211	.007	.153	+.051	6.21	3.52
	7	Hydrolysate	.218	.019	.151	+.048		
	8		.207	.011	.143	+.053		
		Avg	.212	.012	.149	+.051		
5	9	Milk	.197	.043	.142	+.012	6.06	3.73
	10		.198	.023	.100	+.075		
	11		.192	.011	.108	+.073		
		Avg	.196	.026	.117	+.053		
6	12	Acid	.203	.005	.253	—.055	6.65	3.57
	13	Hydrolysate	.214	.012	.199	+.003		
	14		.214	.012	.208	—.006		
		Avg	.210	.010	.220	—.019		
7	15	Enzymic	.204	.013	.202	—.011	6.53	3.57
	16	Hydrolysate	.206	.009	.139	+.058		
	17		.207	.016	.155	+.036	6.18	3.30
		Avg	.206	.013	.165	+.028		

human (*cf.* our earlier report),[2] as well as to determine the relative value of hydrolyzed and unhydrolyzed protein in the maintenance of nitrogen equilibrium.

A diet complete in nutritive elements was prepared in the form of a thin starch paste, divided into 7 portions, and given by gravity, through the gastrostomy opening. The formula for the feeding was: Water *ca.* 800, corn starch 40, dextrose 55, Dextri-Maltose 55, olive oil 55, enzymic or acid hydrolyzed casein 68, yeast 2, NaCl 5, ferrous ammonium sulfate 0.3, dibasic calcium phosphate 1 g, nicotinic acid 40, riboflavin 2, thiamine 6, ascorbic 100 mg and Oleum Percomorphum 20 drops. Using a mechanical stirrer, the water-soluble substances (except vitamins) were first dissolved in the hot water, starch added to gelatinization, and the two oily substances stirred in. The vitamins were added to the almost cool suspension. The formula with milk was made by substituting 692 cc of evaporated milk for the olive oil, protein hydrolysate, calcium phosphate salt, and most of the water. Urine was collected in the usual manner and an s.s. enema in the morning constituted the fecal collec-

2 Cox, W. M., Jr., and Mueller, A. J., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 658.

tion. Three nitrogenous supplements were given: milk, casein hydrolyzed by enzymes* and by acid.†

Daily balances for 6 periods of 3 days each were accomplished before the patient was discharged. Three periods were supplemented with enzymic casein hydrolysate, 2 with milk, and one with the acid hydrolysate (Table I). Nitrogen retention was initially avid, but with time more normal retentions were observed. Thus the 3 periods on the enzymic hydrolysate (level 0.2 g N per kilo) showed decreasing retentions of +.080, +.051, and +.028 g per kilo body weight daily. The 2 periods with milk gave values of +.068 and +.053. These are all exceptionally high retention figures and express the intensity of the patient's need. The 2 supplements are apparently equally effective as nitrogen sources.

The effect of the tryptophane-deficient hydrolysate is of principal interest. The first day of supplementation resulted in a very large urinary nitrogen loss—considerably greater even than the nitrogen intake. The volume of urine for the day was double the usual excretion, and for the period was greater than the previous periods by an average of 700 cc daily. This reversal followed 12 days of large positive retention, and since there was no change in fluid intake, must be attributed to the incompleteness of the acid hydrolysate. Severe loss did not continue, however, since on the second and third day of the period a small positive and small negative balance occurred. These data suggest that the body was able to adjust itself to partially use the deficient mixture. The small net loss, for the 3-day period, —.019 g per kilo, is considerably less than the loss during complete protein starvation (Period 1), and confirms similar observations in rats[2] that even an incomplete source of nitrogen is preferable to no nitrogen at all.

Summary. An enzymic digest of casein may be as effective as milk protein in promoting nitrogen retention, while an acid hydrolysate deficient in tryptophane will not effect nitrogen equilibrium.

* Hydrolyzed with fresh pancreas,[3] known under the trade name of Amigen.

† This has previously been shown adequate for growth in rats when supplemented with 2% tryptophane.

[3] Mueller, A. J., Kemmerer, K. S., Cox, W. M., Jr., and Barnes, S. T., *J. Biol. Chem.*, 1940, **134**, 573.

13950 P

Effects of Lesions of the Periaqueductal Gray Matter in the Cat*

PERCIVAL BAILEY AND EDWARD W. DAVIS.

From the Department of Neurology and Neurological Surgery, University of Illinois College of Medicine.

The great epidemic of lethargic encephalitis directed the attention of neurologists to the region of the midbrain and hypothalamus. A long series of experimental and pathological studies has demonstrated the importance of the gray masses which surround the inferior and posterior parts of the third ventricle, but the posterior extension of the juxta-ventricular gray matter, which surrounds the aqueduct of Sylvius in a cylinder of considerable size, has been neglected. This periaqueductal gray matter is of such a shape, and so located, as to make its destruction very difficult by the usual means—such as the Horsley-Clarke machine—without such extensive damage to neighboring structures as to obscure the interpretation of results.

After much experimentation we developed an electrode which could be inserted by an opening in the occipitoatlantoid ligament

* Aided by a grant from the American Medical Association.

through the fourth ventricle into the aqueduct and, in this manner, made electrolytic lesions confined to the periaqueductal gray matter of the cat. During the process of development of an electrode which would follow the aqueduct we produced numerous lesions, in all parts of the midbrain, which served as controls since in none of them did the symptoms appear which regularly follow destruction of the periaqueductal gray matter. The lesions were made with an electrolytic apparatus, built for us by Mr. Craig Goodwin, which delivers a constant amperage regardless of variations in the resistance. The location of the lesion was, in each case, verified by serial sections stained alternately with the methods of Nissl and Weil.

When the electrode is merely inserted into the aqueduct, without making an electrolytic lesion, the subsequent behavior of the animals is entirely normal. When the electrode jumps the aqueduct and an electrolytic lesion is made outside the periaqueductal gray matter only alterations in motor performance are noted. If the electrode lies too far forward, and there is destruction of the gray matter in the posterior part of the third ventricle, the cats are catatonic. When, however, the electrode lies accurately in the aqueduct, and the lesion is confined to the periaqueductal gray matter, very striking alterations in the behavior of the animals result.

If the lesion is slight the cats, on awakening from the anesthetic, become very wild. They stare vacantly into space, with pupils widely dilated, and spit, snarl, mew and strike as though seeing imaginary menaces. At the same time they pay no attention to actual objects in their environment. This state may last for a day or so and disappear leaving a cat with apparently normal behavior.

If the lesion is somewhat more destructive the above mentioned behavior is transitory and poorly developed, passing rapidly in a few hours into the state to be described next.

If the lesion is extensive, the cats lie inert, silent and flaccid as a wet rag. They never again show any interest in food, but must be nourished by stomach tube. They have no spontaneous activity. They will sometimes swallow if milk is allowed to run down in the back of the throat. Irritation which would cause immediate outcry from a normal cat provokes only feeble movements of the head or limbs. The pupils react promptly to light.

If the animal survives for a few days it begins to right itself, first the head and then the entire body. Placing reactions are elicited normally. It may even walk slowly about on occasion if stimulated, but has no spontaneous activity, attends to nothing in its environment and never feeds itself.

The behavior of these animals with destructive lesions of the periaqueductal gray matter resembles strikingly the syndrome seen in human patients after lesions in the brain-stem, described by Bailey, Buchanan and Bucy[1] as arrest of consciousness and by Cairns, Oldfield, Pennybacker and Whitteridge[2] as akinetic mutism.

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1 Bailey, P., Buchanan, D. N., and Bucy, P., *Intracranial Tumors of Infancy and Childhood*, Univ. of Chicago Press, 1939, p. 250.

2 Cairns, H., Oldfield, R. C., Pennybacker, J. B., and Whitteridge, D., *Brain*, 1941, **64**, 273.

13951 P

The Syndrome of Obstinate Progression in the Cat*

PERCIVAL BAILEY AND EDWARD W. DAVIS.

From the Department of Neurology and Neurological Surgery, University of Illinois College of Medicine.

For some time we have been intrigued by a large cellular mass in the brainstem known as the nucleus interpeduncularis. Gudden[1] noted that "Ueber seine physiologische Bedeutung is so veil wie nichts bekannt." The statement is equally true today. So we decided to make some lesions in it with the aid of the Horsley-Clarke apparatus to see what would result. Judging from its known anatomical connections we expected some alteration of behavior relating to food. Instead there resulted a most amazing behavioral disturbance which we have decided to call the syndrome of obstinate progression.

As soon as the cats recover from the anesthetic they begin to progress obstinately forward, making a peculiar low cry, and will turn aside for no obstacle. If such a cat is in a cage he will thrust his head into the corner and push with all his might until exhausted. He will rub off all the hair and macerate the scalp. If put down on the floor he will start forward and continue directly ahead until he meets an obstacle. He will never turn aside from any obstacle but continue to push his head against it until it gives way or he falls and, by accident, gets a start in another direction. If he is on a table-top he will walk directly ahead beyond the end and fall sprawling to the floor. If the door of his cage is opened he will walk directly out in the same manner and fall. This behavior continues as long as the animal lives, usually about 3 days. He shows no tendency to follow the observer and attends to nothing in his environment, but will eat if he is held with his nose in the neighborhood of food.

In 8 instances of destruction of the interpeduncular nucleus this obstinate progression has developed more or less strongly and never have we observed it when the nucleus was missed. Moreover, in all the lesions which Ranson and his pupils have made in other regions of the pons and midbrain, never was this striking behavior described. In all our cases the location of the lesion has been determined by serial sections stained alternately by the methods of Nissl and Weil.

The only recent description of this syndrome which we have found is that of Mettler[2] who produced it by removal of the frontal lobes of the cerebrum, including the heads of the caudate nuclei. Since removal of the frontal lobes less the caudate nuclei does not produce this abnormal behavior Mettler is inclined to relate it to the destruction of the caudate nuclei. It is interesting to note that Magendie[3] produced violent forward movements by extirpation of both "corps striés" in acute experiments on rabbits.

We are not familiar with any known anatomical or physiological relationship between the caudate nuclei and the interpeduncular nucleus. Since, in none of our cats, was the lesion strictly confined to the interpeduncular nucleus we have avoided calling this abnormal behavior the syndrome of the interpeduncular nucleus, pending the production of more sharply localized lesions, but we felt that it was worthwhile to record its production from a region so far away from the caudate nuclei.

* This investigation was aided by a grant from the American Medical Association.

1 Gudden, B. von, *Arch. f. Psych. u. Nerven krankh.*, 1881, **11**, 424.

2 Mettler, F. A., *Research Pub. Assn. Res. in Nerv. and Ment. Dis.*, 1942, **21**, 150.

3 Magendie, F., *Leçons sur les fonctions et les maladies du système nerveux*, Paris, Lecaplain, 1841.

13952

The Direct Mammotrophic Action of Lactogenic Hormone*

Wm. R. Lyons.

From the Division of Anatomy, University of California Medical School, Berkeley.

In 1937 during a discussion of methods used for assaying the hypophysial lactogenic hormone,[1] the writer spoke of having induced localized, sector lactation in rabbits by intraductal administration of that hormone. It had been shown earlier,[2] that one could compare at least two lactogenic preparations by injecting them locally over the crop sac areas of pigeons. In the New Zealand White rabbits used by us there are usually 8 nipples, each of which has on an average 6 main galactophores emptying to the exterior. Therefore, 48 different sectors in a single animal are available for local assays of the mammotrophic principle. Further study of this problem has revealed that considerable variability in reaction to lactogenic hormone exists between different sectors, undoubtedly due to such variables as (1) the area over which a given volume of hormone is spread (2) the ratio of secretory to duct tissue, (3) the speed and degree of absorption, (4) the blood supply, (5) the amount of reflux, (6) plugs formed, or non-patent areas in some sectors, (7) ruptures occurring in certain sectors with small capacities, (8) cysts or sacculated lobules proximal to the nipple in which the bulk of the injected fluid may accumulate. Because of the large number of sectors in a single rabbit, and because the minimal effective local dose is slightly less than 1 I.U. as compared with the minimal effective systemic dose of about 75 I.U., some of these variables may be controlled by injecting at least one sector in each gland and using for assay purposes only those sectors showing the least difference in capacity, area, uniformity of fluid distribution, etc.

In preliminary experiments it had been possible to cause limited lactation by local introduction of lactogenic hormone even in virgin rabbits with relatively few alveoli on a good duct system. Sector lactation was also obtained in both male and female rabbits pre-treated with estrone-progesterone combinations and in pseudo-pregnant does. In the present series, virgin rabbits, 4 to 5 months old, hysterectomized and oöphorectomized in connection with a separate investigation were used. They were injected subcutaneously 5 days weekly (Monday through Friday) for 4 weeks with 20 μg of estrone and 1 mg of progesterone, separately administered in 0.1 cc of sesame oil. Since the mammary glands of most of these rabbits already showed some duct development at the time of oöphorectomy, 4 weeks' treatment with estrone and progesterone was enough to cause good prolactational growth.

On the third day after the last injections of estrone and progesterone, the rabbits were injected intravenously with 200 mg of nembutal and the hair shaved from the mammary region. With the aid of a binocular-dissecting microscope, test or control solutions were injected into individual sectors using a 1.0 cc tuberculin syringe and a 27 gauge hypodermic needle cut down to about 1.0 cm in length and without a bevel. First, however, the skin and underlying mammary gland were squeezed gently between the thumbs and fingers in such a way as to press out through the nipple pores any accumulated fluid. In the estrone-progesterone treated animals, this was usually of a clear serous nature, but occasionally was slightly turbid. The keratin scale was removed from the nipple with moist cotton and any plugs found occluding the ducts gently massaged to the exterior. Sectors from which a few drops of fluid could be squeezed without trouble were found best

* This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

1 Lyons, W. R., *Cold Spring Harbor Symposia on Quant. Biol.*, 1937, **5**, 197.

2 Lyons, W. R., and Page, E., Proc. Soc. Exp. Biol. and Med., 1935, **32**, 1049.

able to receive and retain the injected hormone. If the needle pierced the duct epithelium, this was revealed by a uniform accumulation of fluid around the base of the nipple before 0.1 cc had been injected. When this happened another duct was tried, preferably in another nipple. If a plug had formed in the main duct or if the duct happened not to be completely canalized, the injection fluid accumulated before 0.1 cc was injected between the nipple and the block. In this event, such a sector was also abandoned. Care in the preliminary emptying of a sector, cleaning the nipple, and especially in introducing the needle to the proper depth, obviates this trouble. In the tests reported herein, only one sector was used for a single dosage of hormone, but in most instances one or more sectors were injected with a control solution of 2% butanol (the concentration used in preserving the hormone). It had been learned that control sectors only lactated when a test sector had received upwards of 1.0 mg of hormone. In such instances, all of the glands lactated, thus indicating a general systemic effect.

TABLE I.

Mammary Sector Reactions in Oöphorectomized Virgin Rabbits Pre-treated with Estrone and Progesterone and Then Injected Intraductally with Varying Amounts of Lactogenic Hormone in 1.0 cc of a 2% Butanol Solution.*

No. of animals	Dosage of hormone		Reactions	
	mg	Approx. I.U.	—	+
6	.012	0.37	6	0
6	.025	0.75	3	3
6	.05	1.5	5	1
6	.1	3.0	0	6
6	.2	6.0	0	6

*At least one other gland of each rabbit was injected with a control solution of butanol and in no instance was lactation induced.

The rapid absorption of the injected water of control and test solutions was readily observed. The gland could be seen to become distended when 0.5-1.0 cc was injected and within a few minutes, the sector appeared normal again. Milk has been expressed from a test sector as early as 24 hr after the injection. It was found, however, that the amount of milk formed after a single injection, increased until about the fifth day and that sometimes, the accumulated milk remained until the tenth day or longer. Thus, although a shorter test could be used it was thought better in this study to judge the reaction on the fifth day when the positive effect of border-line doses was more easily determined. The accompanying table contains the data from a typical experiment in which 30 rabbits were used. One sector of a single mammary gland in each rabbit was injected with 1.0 cc of a 2% butanol solution containing a given amount of lactogenic hormone containing approximately 30 I.U. per milligram. Five different levels ranging from 0.012 to 0.2 mg were tested in each case in 6 different rabbits. Each rabbit provided its own control glands, at least one of which was injected with 2% butanol. The lactogenic hormone was given in a single injection, 3 days after the last estrone-progesterone treatment. Attempts were made to express milk from the test and control glands every 24 hr, beginning 48 hr after the hormone was injected. A reaction was considered positive if by the fifth day, milk could be expressed from a sector. In this experiment, confirmation was made in some cases by cutting back a skin flap and judging directly the reaction in the gland with the help of a binocular dissecting microscope. This provided an opportunity for taking specimens for histologic study and also permitted comparison of the injected sector with adjacent uninjected ones.

Results and Discussion. As shown in Table I, all of the sectors injected with 6 and 3 I.U. of lactogenic hormone lactated. Five of 6 on the 1.5 I.U. level, 3 of 6 on the 0.75 I.U. level and none of the 6 on the 0.37 I.U. level gave a positive response. None of the control sectors lactated. It was always possible to express milk from the positive sectors within 48 hr of the injection of the higher doses. With the border-line dose of 25 μg the secretion was turbid at the 48th hr, but changed to milk between the third and fifth day in half of the animals. It was possible to observe through the skin the gradual accumulation of milk in the injected sectors only. That the hormonal effect lasted for several days was shown by expressing the

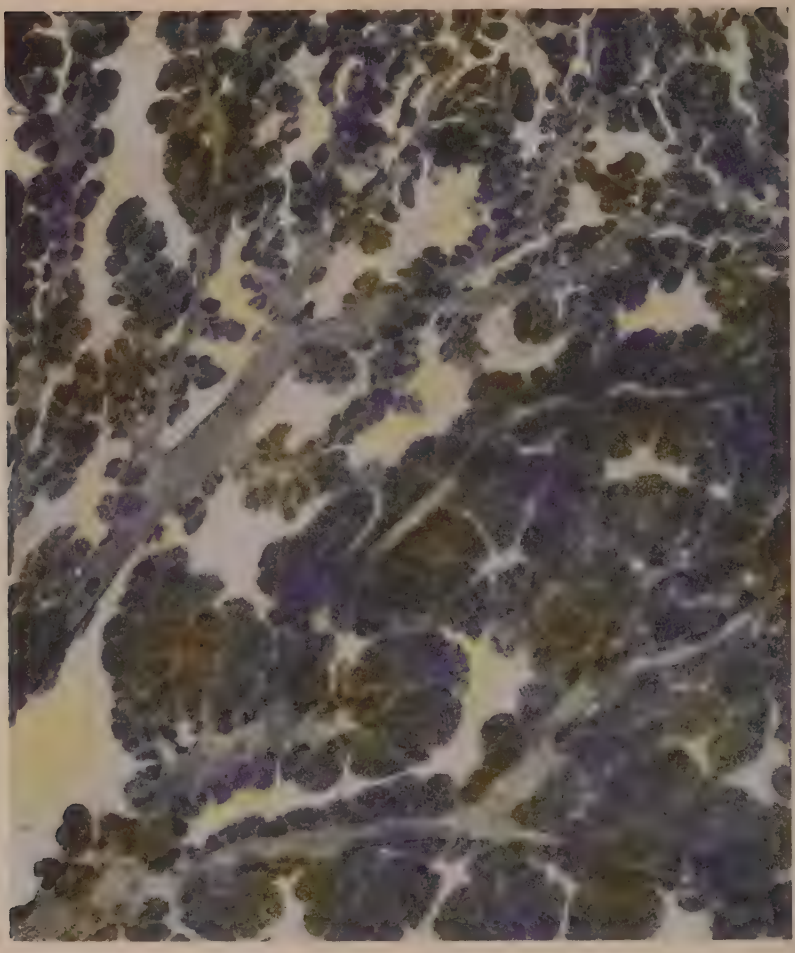

FIG. 1.

Two sectors of a mammary gland of an oöphorectomized rabbit, pre-treated with estrone and progesterone, and then given 1.0 cc (3 I.U.) of lactogenic hormone into the main duct (lower left) of the bottom sector. Milk could be expressed from this sector 48 hours after the injection, and the gland was removed 24 hours later, fixed in formol stained in alum-carmine and cleared in methyl salicylate. No other sector of this or other glands lactated. The prolactational growth seen in the upper control sector is in striking contrast to the additional, lactational growth induced by the local administration of lactogenic hormone. × 6.

milk from these sectors daily and observing its re-accumulation for at least a week. Whether this be due to an initial "trigger-action" of the hormone, or to effective residual amounts of it continuing to bathe the secretory units cannot be decided at this time.

Fig. 1 shows representative areas of 2 separate sectors in a mammary gland of a rabbit pre-treated with estrone and progesterone as specified above and then given 0.1 mg (approximately 3 I.U.) of lactogenic hormone in 1.0 cc. The large duct in the lower left corner is the main galactophore of the injected sector. As may be seen, the lower or injected sector was in full lactation when this gland was removed 3 days after the injection was made. The upper or control

sector shows only the prolactational growth induced by the estrone-progesterone treatment. Before sacrificing the animal it was possible, by pressing the area of the gland shown in the figure, to observe milk exude from the injected pore in contrast to a clear serous fluid from the upper sector. However, this manipulation was quite unnecessary since the contrast between the positive and negative sectors was even more striking as seen through the skin than in the accompanying figure.

Histologic study of the glands used in these experiments was especially interesting because it was possible to compare in a single section lactating and non-lactating lobules from adjoining sectors. Such sections were found valuable in contrasting the prolactational growth induced by the estrone and progesterone treatment, and the additional, lactational growth brought about by lactogenic hormone. That the latter should be considered a mammary growth-promoting hormone is maintained for the following reasons: (1) the number of epithelial cells forming the circumference of alveoli growing under the influence of lactogenic hormone is several-fold that of the control alveoli; (2) a large number of the alveolar cells are cast off in the formation of the first milk and are replaced by newly-proliferating cells; (3) mitotic figures may be observed in the alveolar epithelium during the lactational growth phase, and the probability of amitotic proliferation must also be entertained; (4) there are not only more cells per alveolus during this growth phase, but they are, at certain stages of their cycle, larger than the cells of the resting alveoli; (5) when full lactation has set in, the secretory cells have to be constantly renewed either in their entirety or—as is more usually the case—in their supra-nuclear cytoplasm only. The directly-stimulated sectors were also studied in their regressive stages and the decrease in the number and size of the secretory cells after the withdrawal of the hormonal influence was just as striking as their increase when hormone was added.

Summary. Thirty oöphorectomized, virgin rabbits, injected subcutaneously 5 days weekly for 4 weeks with 200 I.U. of estrone and 1 I.U. of progesterone showed prolactational mammary growth approximating that of a 3-weeks' pregnant rabbit. Three days after the last of these injections, 5 different levels of lactogenic hormone were tested on groups of 6 rabbits. The hormone was given intraductally in such a way that an individual sector in each animal received a constant volume of fluid (1.0 cc). Of the different doses of hormone tested, 6 and 3 I.U. caused localized sector lactation in all 6 animals; 1.5 I.U. in 5 of 6; and 0.75 I.U. in 3 of 6 animals. None of the 6 animals injected with 0.37 I.U. lactated; nor did the uninjected or control-injected sectors in any animal lactate. This experiment demonstrates for the first time that the hypophysial lactogenic hormone has a direct mammotrophic effect just as it was previously shown to have a direct crop sac stimulating action. With all other factors essentially equal in any 2 adjoining sectors in a mammary gland, the mere bathing of the parenchyma of one with 25 μg of this purified protein brought about the complex cell-growth and cell-renewal processes of lactation, whereas the introduction of a control solution or no treatment whatsoever, caused no detectable change in the other.

The estrone and progesterone used in this study were generously supplied by Parke, Davis and Company, Detroit, Mich.

All preliminary manuscripts are indicated by the letter P after the number of the article

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